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From the Psychiatric Clinic of the Rigshospital, Copenhagen.

Changes in the Respiratory Quotient after Administration of Insulin and Glucose to Human Subjects on a High and on a Low Carbohydrate Diet.

By

KNUD LUNDBÆK.

Received 12 October 1943.

By insulin shock treatment of psychotic patients favourable conditions have been created for many different investigations on the effect of insulin on man. Partly the carbohydrate metabolism of the patients is generally normal and they can therefore function as normal subjects, partly it is possible during insulin shock treatment to give highly variable insulin doses, especially much larger doses than can otherwise be administered. And already now numerous physiological insulin investigations are, in fact, available from psychiatric departments.

In the present work an account will be given of a series of investigations on the respiratory quotient after administration of large and small doses of insulin and after administration of glucose. In other publications a series of other results of the same investigations will be treated, viz. the fasting values of blood sugar, RQ, and alveolar CO_2 tension on a high and on a low carbohydrate diet; the sensitivity of the respiratory centre, and the pH of the blood during insulin treatment. The various experimental results have already been published with full details of the experiments in a Danish thesis for the doctorate (LUNDBÆK 1943).

On the suitability of psychotic patients for physiological investigations on the carbohydrate metabolism. The subjects were patients from the

Psychiatric Clinic of the Rigshospital, most of them fresh cases of schizophrenia. (There were, however, no katatonic or "tense" patients among the subjects). Such patients usually have a normal basal metabolism and show no symptoms of abnormalities in their carbohydrate metabolism, as will appear from the works of FISCHER (1928, 1932) and HENRY (1929) and from an examination of the material of REITER (1925) and LANGFELDT (1926). The subjects of the present investigation all showed a normal basal metabolism. None of them suffered from glucosuria, and all exhibited normal glucose tolerance curves with the exception of a single patient whose glucose tolerance curve was of normal height but with a somewhat protracted course. This patient, however showed no difference in the results from the other subjects.

As of special interest for the problems here discussed it may besides be mentioned that the administration of large doses of insulin, as in the insulin shock treatment, does not change the well known relation between the content of carbohydrate in the diet and the shape of the glucose and insulin tolerance curve (LUNDBÆK and MAGNUSSEN, 1940).

The Object of the Investigations.

It is curious to note the small number and extent of the investigations to be found in the literature on the effect of insulin on the RQ, especially in man. In animal experiments various circumstances have rendered doubtful the interpretation of the values obtained. With human subjects the most extensive experiments by far are those of HOLTEN (1929) but this author only determined the RQ before and 2 hours after administration of small doses of insulin so that no impression can be gained from his work of the *course* of the "RQ curve". The object of the present experiments was to determine the effect of insulin on the RQ of man, especially the relation between the blood sugar curve and the RQ curve. It was clear, therefore, that dynamic methods of investigation must be used, with frequent determinations, while the reality of the RQ must as far as possible be assured, and finally there must be a possibility of the administration of very variable doses of insulin. In view of the recent interest in the bearing of the carbohydrate content of the diet on the carbohydrate metabolism it seemed natural to include this problem and make investigations on a fixed diet either rich or poor in carbohydrates, so as to find out a possible difference in the carbohydrate metabolism on these two diets, and the peripheral or hepatic localisation of this difference, if present. — As most of the previous investigations on the significance of the preceding diet have been made by means of the administration

of glucose, a short series of glucose experiments have also been carried out with exactly the same technique as in the insulin experiments.

Previous Investigations on the RQ and Blood Sugar after Administration of Insulin and Glucose on Various Diets.

The significance of the carbohydrate content of the diet for the carbohydrate metabolism has been known for many years but was only taken up for closer study by HIMSWORTH in a long series of works (1933—39). He showed that the height and length of the glucose tolerance curve is in inverse ratio to the amount of carbohydrate administered in the preceding period, and that it was only the carbohydrate content of the diet which had any bearing on this variation, not fat, protein, or total calories. That this variation was not, or at any rate not alone, due to differences in the secreted amount of insulin on the different diets appeared from the fact that also the blood sugar curve after intravenous injection of insulin showed a distinct dependence on the carbohydrate content of the diet: the more carbohydrate in the diet the steeper was the fall of the insulin tolerance curve. HIMSWORTH called this phenomenon the insulin sensitivity. That a change must, however, also be supposed to take place in the insulin secretion itself upon a change in diet appears from HAIST, RIDOUT and BEST's demonstration (1939) of a lower insulin content in the pancreas after a diet poor in carbohydrates.

The difference in the shape of the blood sugar curve upon glucose or insulin administration on diets with a different carbohydrate content must be determined either by a difference in the inflow of glucose from the liver or by a difference in the "outflow", that is to say in the peripheral emigration from blood to cells. SOSKIN and MIRSKY (1935—36), by experiments on eviscerated animals, arrived at the conclusion that the difference must lie in a change in the output of glucose from the liver upon a change in diet. HIMSWORTH, on the other hand, is most inclined to regard this difference as the result of a variation in the peripheral combustion of sugar, basing this opinion on previous investigations which have shown a reduced power of sugar combustion (absence of a rise in the RQ upon administration of carbohydrate) after a period of fasting (DANN and CHAMBERS, 1930). On a critical

perusal of the literature on this problem, however, (JOHANSSON, 1908; CORI and CORI, 1928; HINES, BOYD and LEESE, 1929; SPENCER and MCCLELLAN, 1932; CHANDLER and CHAMBERS, 1938; and especially JOHNSTON, SHELDON and NEWBURG, 1939) one gains the impression that the "reduced rate of carbohydrate combustion" only occurs after fasting for some time or after a heavy and prolonged reduction in the carbohydrate of the diet: In case of a milder carbohydrate deficiency such a reduction does not seem to occur, despite the fact that the characteristics of the shape of the glucose tolerance curve which set in promptly after a change in the carbohydrate content of the diet (ADELSBERGER and PORGES, 1926) are present. Hence, according to this, the *degree* of carbohydrate deficiency should be of great significance. "A reduced rate of carbohydrate combustion" would be a result of extremely abnormal metabolic conditions.

The possibility existed, however, that a slight reduction in the rate of carbohydrate combustion in mild degrees of carbohydrate deficiency could be compensated by the higher blood sugar values obtained by the administration of carbohydrate on such a diet. The emigration of glucose from blood to cells and therewith the glucose combustion is, as we know, dependent on 1) the blood sugar concentration and 2) the insulin concentration (and possibly the insulin sensitivity). If with HIMSWORTH we imagine that the primary change upon transition to a low carbohydrate diet is a reduction of the peripheral emigration of glucose from blood to cells, it might be possible that this would secondarily involve a "blood-sugar stasis" (revealing itself in the raised glucose tolerance curves) which in mild cases would be sufficient to compensate the reduced rate of carbohydrate combustion. If such a *self-regulation* takes place, it would manifest itself by the same rise in the RQ after administration of glucose on diets with a varying carbohydrate content.

While thus there are a good many investigations on the change in the RQ after administration of glucose on various forms of diets, such investigations on conditions after administration of insulin seem hardly to be found in the literature, though, as is well known, characteristic differences in the blood sugar curve can also be pointed out after administration of insulin on different diets.

Of investigations on the effect of insulin on the RQ — *without* taking account of the preceding diet —, there are, however, a

good many though, as already mentioned, not as many by far as might be expected.

The first investigations, which were made on mice, guinea-pigs, rabbits, and dogs, showed various results: partly a rise partly a fall of the RQ after administration of insulin (DUDLEY, LAIDLAW, TREVAN and BOOCK, 1923; DICKSON, EADIE, MACLEOD and PEMBER, 1924). Later investigators have, however, most frequently been able to demonstrate a rise in the RQ (GABBE, 1924; KROGH and BRANDT REHBERG, 1925; BOOTHBY and WEISS, 1925; HAWLEY and MURLIN, 1925—26; REISS and WEISS, 1926). Experiments with human subjects have been made by KELLAWAY and HUGHES (1923); LYMAN, NICHOLLS and MCCANN (1923); HOLTEN (1929); and REITER (1933). All these authors find a rise of the RQ after administration of insulin to human subjects.

Information about the relation between the magnitude of the rise in the RQ and the insulin dosage only appears in a few works. GABBE found that small doses of insulin caused a small and *brief* rise in the RQ. From the work of REISS and WEISS it appears that very large doses gave a smaller rise than smaller doses. On human subjects HOLTEN found no relation between the depth of the fall of blood sugar and the height of the rise in the RQ. The relation between the blood sugar curve and the *course and shape* of the RQ curve has not been given special attention. Only KROGH and BRANDT REHBERG state: "When the blood sugar is reduced to a very low level by the insulin, the quotient appears to fall, but our observations are not sufficiently numerous to establish this as a fact."

The reality of the RQ values found has been questioned by authors who have demonstrated a simultaneous fall in the CO_2 content of the blood in the animals examined (BOOTHBY and WEISS, 1925; LUNDE SVEINSSON, 1941). In man this feature has not previously been investigated. In another paper (LUNDBÆK, 1944) it has been shown that in human subjects there does not usually occur any blowing off of CO_2 in the quiet phase of the insulin effect.

Technique, Insulin Dosage, Diet, etc.

In all experiments determinations of the ventilaton, O_2 assimilation, CO_2 excretion, alveolar CO_2 tension, and blood sugar have been made at regular intervals — about every half hour — after administra-

tion of insulin or glucose. In most cases the blood pressure has also been examined and the rectal temperature taken at similar intervals.

The respiratory functions were examined by means of one of the ordinary respiration apparatus of the open type. The ventilation was measured by means of a gas meter. The respiratory movements were registered continuously. The alveolar CO_2 tension was determined on air samples taken refractorily according to the method of LINDHARD (1911). The air samples were analysed in a Haldane apparatus. The blood sugar was determined in double samples according to HAGEDORN-NORMAN JENSEN. — The calculation of the values found in the respiration experiments was made in the usual way. The calorie production was calculated by means of ZUNZ and SCHUMBURG's tables for the nonprotein quotient without taking into account the small error thereby produced.

The experiments were made on a fixed high or low carbohydrate diet. This diet, the fasting values of the RQ, the blood sugar, and the alveolar CO_2 tension are discussed elsewhere (LUNDBÆK 1944). Here it need only be mentioned that the high carbohydrate diet contained an average of 512 g carbohydrate daily, the low diet 135 g.

Insulin dosage and amount of glucose. "Large" and "small" doses of insulin were given. By large doses are meant such as usually caused deep hypoglycemic coma in the patient in the course of 3—4 hours. These doses differ very much individually, ranging between 32 and 544 int. units., as a rule, however, the dose was 100—200 units. By small doses are meant such as did not give rise to hypoglycemic symptoms in the patients. These doses varied from 8 to 32 units, most frequently they were 16 units. In all cases the insulin was given intramuscularly. — After large doses of insulin the average fall in blood sugar half an hour after the injection was about 40 mg %, after small doses 20 mg %. There was a tendency to a HIMSWORTH effect, but it has not been possible to demonstrate any sure statistic difference in the blood sugar curve on the two diets, presumably on account of the size of the doses and the intramuscular mode of administration. — In the glucose experiments 70 g glucose were given by mouth in all cases.

The experiments lasted 2—4 hours. After large doses of insulin it was often necessary to break off the experiment after a couple of hours as the respiration at that time grew very irregular and made it impossible to obtain a reliable RQ. After small doses and after administration of glucose the experiments always lasted 4 hours.

The reality of the RQ. An absolutely steady state, as is desirable for RQ determinations, is rarely attained after administration of insulin or glucose. In the present insulin experiments a rise most frequently took place in the ventilation as well as in the alveolar CO_2 tension. The changes, however, were so small and smooth that there has hardly been any considerable falsification of the RQ. All experiments in which abrupt changes in the reverse direction in the ventilation and the alveolar CO_2 tension occurred were cancelled. The frequently occurring reduction in the sensitivity of the respiratory centre will, however, presumably in some slight degree tend to give too low RQ rises. — It must thus

be assumed that the RQ values stated were, though not as certain as can be achieved in experiments on trained subjects under absolute standard conditions, at any rate sufficiently accurate to be used as a basis for the estimation of the changes in the carbohydrate metabolism taking place after administration of insulin and glucose (see also LUNDBÆK, 1944).

Results.

The General Course of the RQ Curve after Large and Small Doses of Insulin.

Figs 1—2 show *summation curves of the rise in RQ and the* fall in blood sugar for all experiments after large and small doses of insulin, not taking into account the preceding diet. From these curves a rough idea can be gained of the usual or "typical" course of the RQ curve.

The characteristic feature of both curves is that a rise takes place in the RQ, that this rise is transitory, and that it is replaced by a fall to the initial value.

After large doses the rise is high and steep and rapidly reaches its maximum. After small doses the rise is more gradual and the

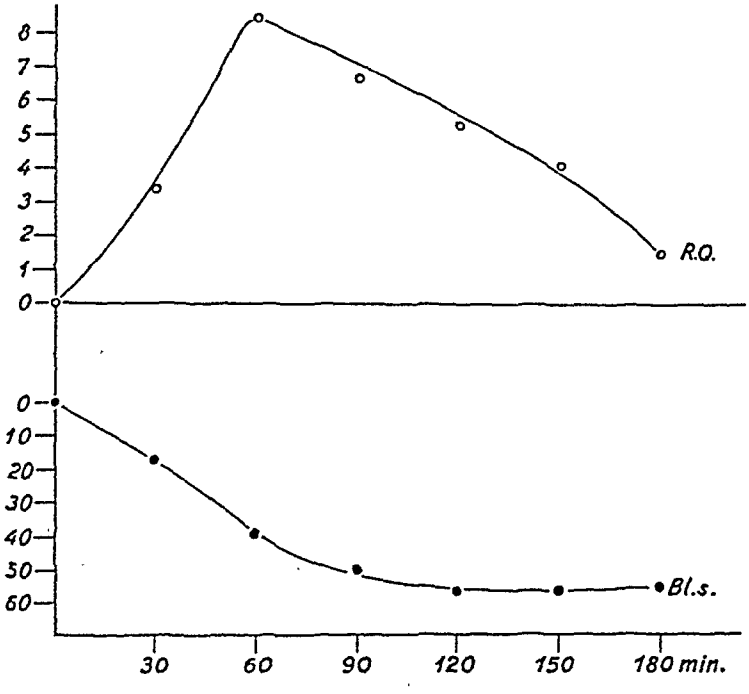

Fig. 1. Rise in RQ (RQ-units = 0.01 RQ) and fall in blood sugar (mg %) after large dose of insulin.

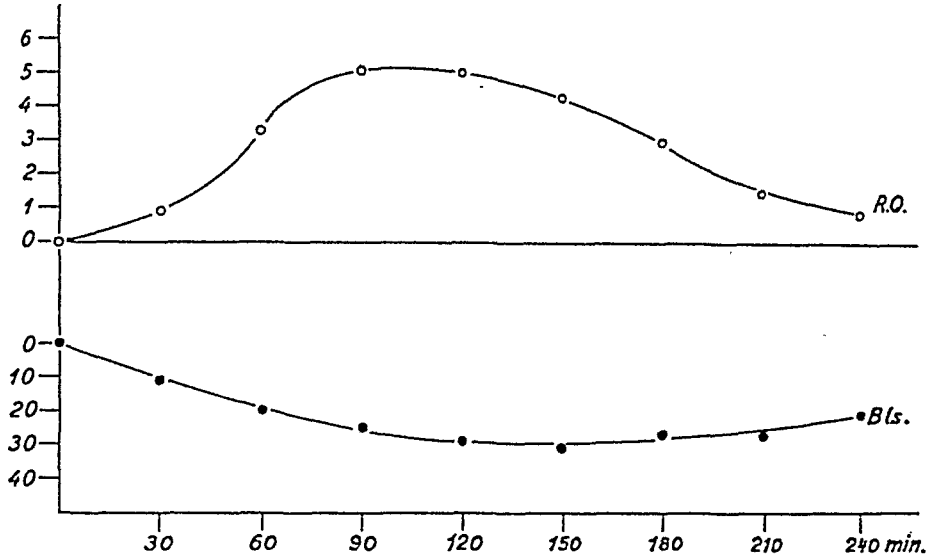

Fig. 2. Rise in RQ (RQ-units = 0.01 RQ) and fall in blood sugar (mg %) after small dose of insulin.

RQ attains its maximum later. After large doses the fall is sudden, while it is quite smooth after small doses. The result is that after large doses the RQ curve shows a peak, while after small doses it exhibits a greater tendency to form a maximal plateau. After small doses the whole process seems to be more protracted, the RQ reaching the initial value later than after large doses. Finally, it is seen that the RQ maximum on both curves approximately coincides with the point on the blood sugar curve where the latter begins to be flattened.

Below, the results will be stated of a closer statistic analysis of the particulars of the RQ curve, just as the problem of diet and the relation between the RQ curve and the blood sugar curve will be discussed in detail.

The Height of the Rise in the RQ.

In a statistic calculation of the individual results of the RQ determinations the average maximal rises in the RQ were calculated as well as the mean error of these averages on the two diets after large and small doses. (In the calculation "RQ units" = = 0.01 RQ have been used.) After large doses a rise of 11.3 ± 1.64 was seen on diets rich in carbohydrates, and a rise of 9.46 ± 0.71 on diets poor in carbohydrates. After small doses

there was a rise of 5.23 ± 0.66 on a high carbohydrate diet, and of 6.94 ± 0.40 on a low carbohydrate diet. There is a significant difference in the effect of large and small doses on both diets, but no certain difference in the influence of the two forms of diets either after large or after small doses.

The Time of the RQ Maximum.

By a similar treatment of the time — i. e. number of minutes after the injection — when the RQ maximum sets in (with a maximal plateau the central point of this has been taken) we arrive at the following results: The RQ maximum was attained after large insulin doses on a high carbohydrate diet after 76.2 ± ± 7.7 minutes, on a low carbohydrate diet after 68.5 ± 6.4 minutes. After small doses the maximum was found on a high carbohydrate diet after 128.1 ± 17.6 minutes, on a low carbohydrate diet after 117.3 ± 4.4 minutes. — There is a significant difference in the effect of large and small doses on both diets, while here too the diets have not had any certain influence on the result in the two dosage groups.

The RQ Curve and the Blood Sugar Curve.

From the summation curves (figs. 1—2) we gain the impression that the RQ maximum after large as well as after small doses occurs simultaneously with incipient flattening of the blood sugar curve. If this is the case, the RQ rising with falling blood sugar and falling again when the blood sugar becomes constant at some level or other, we might expect to find in the individual experiments *either* an approximate coincidence of the time when the RQ begins to fall and a point on the blood sugar curve where the latter passes from steepness into a more or less marked plateau, *or* no fall in the RQ curve with unchanged steepness of the blood sugar curve.

On reviewing the individual experiments the following result is obtained. The above-mentioned coincidence (or absence of fall in the RQ) can be demonstrated in 18 out of 32 experiments after large doses of insulin. 7 experiments were uncertain on account of small irregularities in respiration, too few blood sugar samples, or the like. 7 experiments do not show this coincidence. After small doses the relation is more difficult to estimate but a coincidence like the above-described would seem to occur in 13 out of 16 experi-

ments. In 3 experiments it was not found. — Hence, taking all the experiments together it is seen that the coincidence looked for can be demonstrated in 31 out of 48 experiments. 7 experiments gave no sure result. 10 experiments did not show the coincidence in question.

Hence the result of this survey is not unambiguous. Since, however, it is only in one-fifth of the cases that it has not been possible to demonstrate the coincidence in question, it must be justifiable to conclude that there is presumably a reality behind the oft-demonstrated coincidence between the time when the RQ fall sets in and the time when the blood sugar curve decreases in steepness and passes into a more or less pronounced plateau. If this is the general rule the relation may also be expressed thus: While the blood sugar curve falls the RQ rises. When the blood sugar adjusts itself to a new low level the RQ falls back towards the initial value. This means, then, that the changes in the RQ are dependent on *changes* in the blood sugar, not on the blood sugar level itself. That the latter is not the case, that the fall in the RQ is not due to the low blood sugar level, appears plainly from a consideration of the blood sugar level at the time when the RQ begins to fall. It is seen in the individual cases that this can take place at a blood sugar level of between 10 and 80 mg %. Conversely, in some experiments no fall in the RQ is seen, even though the blood sugar in these has fallen to values between 30 and 50 mg %. That the fall in the RQ should be due to cessation or reduction of the insulin effect is very improbable as far as the large doses are concerned, since the blood sugar is still at a low level, and hypoglycemic symptoms are developing. After the small doses it is true that in some cases a rise in the blood sugar curve would seem to indicate a decreasing insulin effect, but in many other cases no rise in the blood sugar is seen when the RQ begins to fall.

In spite of the lack of uniformity in the results it thus seems not unreasonable in general to explain the fall in the RQ by a reduction or cessation in the fall of the blood sugar.

"Excess Amount of Glucose Consumed Minus Lost Glucose".

In the individual experiments the extra amount of glucose consumed has been calculated from the RQ curves, the O_2 consumption at the different points of time and the percentage share in the total metabolism constituted by the carbohydrate com-

bustion at each single RQ value according to CARPENTER's tables (1939). The lost glucose has been calculated from the total fall in blood sugar in the individual experiments, starting from a distribution of the glucose over an amount of fluid corresponding to 30 % of the body weight. This distribution cannot be accurately determined but must be supposed to be of an order of magnitude of 30 % as the muscle cells are known not to contain glucose (LUNDSGAARD, 1939).

By a statistic calculation of the averages for the "excess amount of glucose consumed minus the lost glucose" and the mean error of these averages we get the following results: After large doses on a high carbohydrate diet: -4.52 ± 1.32 g. After large doses on a diet poor in carbohydrates: -5.08 ± 0.95 g. After small doses on a diet rich in carbohydrates: -2.63 ± 1.76 g. After small doses on a low carbohydrate diet: $+0.93 \pm 0.85$ g.

It appears from the calculations that after large doses of insulin both on high and on low carbohydrate diets 4—5 g glucose not seen to have been consumed seems to have disappeared from the blood. After small doses on a high carbohydrate diet this deficit was smaller, while on a low carbohydrate diet there was no certain deficit.

Glucose Experiments.

Since, as previously mentioned, most of the earlier investigations on the influence of the diet on the carbohydrate metabolism have been carried out with glucose tolerance determinations we have here made a shorter series of experiments with administration of 70 g glucose by mouth. The technique was in other respects the same as in the insulin experiments. The experiments lasted for 4 hours with determination of the RQ, blood sugar, etc. every half hour.

Experiments were made with two subjects. These showed different responses to a change in diet. One of them exhibited a very considerable rise in the glucose tolerance curve upon transition to a low carbohydrate diet, the other only a very small rise. This corresponds to earlier experiences since it is well known that the *degree* of variation at a change of diet differs individually. By determining the excess amount of glucose consumed in 4 hours (according to the above-described principles) and correlating these values with the maximal rises in blood sugar, the result shown in fig. 3 was obtained.

It appears with all clarity that the excess combustion is the greater the higher the glucose tolerance curve is, just as the difference between the two subjects (o) and (+) is seen. — The same result is obtained in a detailed statistic calculation of the individual experimental results.

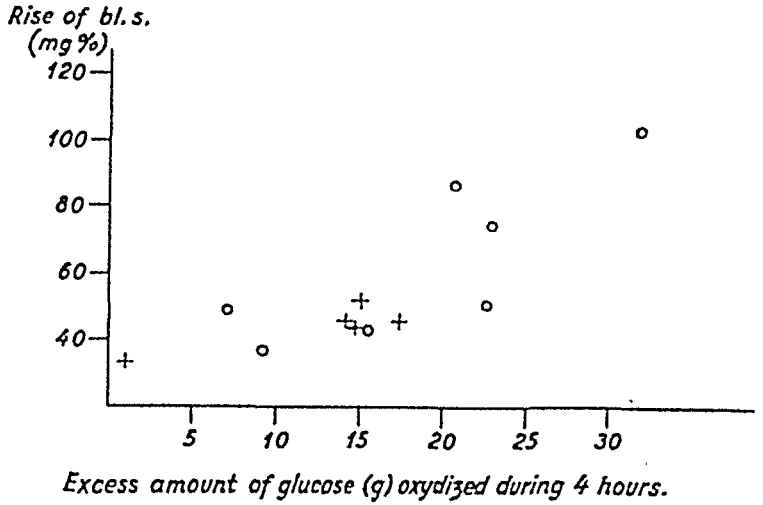

Fig. 3. Correlation between maximal rise in blood sugar and excess amount of glucose consumed in the glucose experiments.

Discussion.

The present insulin experiments give an impression of the *course* taken by the change occurring in the carbohydrate metabolism upon administration of insulin. The whole process seems to last 3—4 hours and consists in a rise followed by a fall in the relative share of the carbohydrates in the total metabolism.

With respect to the relation between the insulin dosage and the rise in the RQ it has not been possible to confirm GABBE's, REISS and WEISS's, and HOLTEN's above-mentioned results, direct proportionality having been demonstrated between the size of the dose and the height of the rise in the RQ, while the rise in the RQ has been rather more protracted after small doses than after large doses.

The correlation between the blood sugar curve and the RQ curve likewise becomes evident from the experiments. It turns out that the RQ rises while the blood sugar falls. When this fall ceases, the RQ generally falls again to the initial value. This

means, then, that the metabolic changes produced by the insulin are secondary to the fall in blood sugar. When the fall in the blood sugar ceases, the combustion of carbohydrates returns to the initial level, even though the insulin effect in itself is still maximal, as will appear from the persistence of the low blood sugar level and, after large doses, from the occurrence of severe hypoglycemic symptoms and coma which, if not interrupted by intervention from without, may lead to death.

The calculation of "excess amount of glucose consumed minus lost glucose" has shown that after large doses and after small doses on a high carbohydrate diet a not inconsiderable amount of glucose not seen to have been oxydized disappears from the blood. This deficit must either be due to a deposition of glucose as glycogen in the muscles or to a lowered glucose output from the liver. — A considerable deposition in the muscles after administration of insulin only seems to take place with a simultaneous administration of large amounts of glucose (BEST, DALE, HOET and MARKS, 1926; BARBOUR, CHAIKOFF, MACLEOD and ORR, 1927). Such a deposition, therefore, can hardly be responsible for the entire deficit found here.

Whether insulin has any effect on the liver and in what, if any, it consists has been subject to numerous investigations and much discussion, but it cannot be said that any conclusive result has been arrived at. Upon investigation of intact animals it is true that a fall in the liver glycogen has most frequently been found after administration of insulin (CORI, 1925; CORKILL, 1930; BODO and NEUWIRTH, 1931; BÜRGER and KOHL, 1935), but the objection has rightly been raised against these experiments that the observed loss of glycogen has probably been caused by a restorative adrenalin secretion (CORKILL, 1930). By using very small insulin doses which did not give rise to hypoglycemic symptoms FRANK, NOTHMANN and HARTMANN (1928) obtained a rise in the liver glycogen on rabbits. Perfusion experiments with isolated liver have especially been carried out by LUNDSGAARD and his pupils (NIELSEN, 1932; LUNDSGAARD, NIELSEN and ØRSKOV, 1936, 1939). In spite of very variable experimental conditions it has never been possible to demonstrate any direct effect of insulin on the glucose output of the perfused liver. — Altogether, however, after a survey of the literature it seems not improbable that insulin in some way or other indirectly causes a reduced glucose output from the normal liver, as it is known to be the case also with

the diabetic liver. How this happens cannot be settled for the present, especially because our knowledge of fat metabolism is so scanty. Actually we do not know whether the peripheral fat combustion consists in a combustion of fatty acids, ketones, or glucose formed by fat. If the latter is entirely or partly the case, a reduced glucose output might simply mean that the part of the glucose output from the liver which "belongs to" the fat metabolism is reduced when the carbohydrate metabolism rises after administration of insulin. For, with the insulin effect, where the total metabolism is practically unaltered and the protein metabolism hardly changes, it is merely a question of a relative shifting of the share of the carbohydrates and the fat in the combustion. (It must here be noted that the doubt expressed in various quarters (inter alia by SOSKIN, 1941) as to the suitability of the total RQ for the estimation of the combustion processes is only justified in cases where there is a conversion, e. g. of fat to sugar, and where this sugar is not at once consumed but deposited in the form of carbohydrates. But in the circumstances here under consideration which hardly differ from "normal" there is no reason to assume anything of the kind.)

It will therefore be reasonable to ascribe at any rate part of the discrepancy found between the excess amount of glucose consumed and the lost glucose to an inhibition of the glucose output of the liver. This inhibition, as was to be expected, proves greater after large than after small doses of insulin.

With respect to the significance of the diet the facts are most clearly revealed in the glucose experiments. It was shown by these that there could be no question of any reduced peripheral oxidation on a low carbohydrate diet (HIMSWORTH, 1939), not even with the previously discussed self-regulation, since the excess combustion proved to be greater the higher the glucose tolerance curve is. We are therefore compelled to assume with SOSKIN and MIRSKY (1935—36) that the difference in the blood sugar curves is due to a liver effect, whether we would express this in terms of a reduction in the inhibitory effect of the endogenous insulin on the glycogenolysis caused by the low carbohydrate diet, or — if we assume that fat is partly or wholly converted into glucose before it is consumed — simply in terms of the predominant fat metabolism in the liver on low carbohydrate diets, owing to which a great deal of the glucose output is in reality a breakdown product of fat.

The insulin experiments are of less importance for the problem of diet, inter alia because, as already mentioned, no certain statistic difference has been obtained in the blood sugar curve on the two diets. — After large doses of insulin no difference was seen in the deficit in the two diets. After small doses there was no deficit on the diet poor in carbohydrates. Even though the difference between the deficits on diets poor and rich in carbohydrates is merely on the verge of statistic significance it will still be natural to explain the absence of a deficit on the low carbohydrate diet just as in the glucose experiments by a reduction of the inhibitory effect of the insulin on the glucose output from the liver, in whatever way this inhibition is supposed to be brought about.

Summary.

A series of investigations has been made on the respiratory quotient after administration of large and small doses of insulin and after administration of glucose on diets rich and poor in carbohydrates.

The RQ curve after administration of insulin is diphasic. During the fall in the blood sugar the RQ rises, when the fall in blood sugar ceases the RQ as a rule goes back to the initial value. It would seem, therefore, that the changes in the RQ should be viewed in relation to the blood sugar *fall* itself.

The rise in the RQ was greater after large than after small doses of insulin. The maximum RQ is attained more rapidly after large than after small doses. These two factors are unaffected by the carbohydrate content of the diet.

In the glucose experiments it is shown that the excess combustion of sugar after administration of glucose is greater the higher is the glucose tolerance curve. It appears from this that a slight reduction of the carbohydrate content of the diet does not reduce the rate of combustion of carbohydrate. The characteristic high and long glucose tolerance curve on a diet poor in carbohydrates must therefore be due to a liver effect.

In the calculation of "excess amount of glucose consumed minus lost glucose" in the insulin experiments it is seen that a not inconsiderable amount of glucose not seen to be oxydized disappears from the blood. The significance of this discrepancy is discussed and the conclusion is drawn that it must at any rate in part be supposed to be due to a reduced output of glucose

from the liver during the insulin effect, even though it is not at present possible to account in detail for the mechanism of this process.

References.

Adelsberger, D., and O. Porges, Klin. Wschr. 1926. *5*. 1451.

Barbour, A. D., I. L. Chaikoff, J. J. R. Macleod, and M. D. Orr, Amer. J. Physiol. 1927. *80*. 243.

Best, C. H., H. H. Dale, J. P. Hoet, and H. P. Marks, Proc. Roy. Soc. Lond. Ser. B. 1926. *100*. 55.

Bodo, R. C., and J. Neuwirth, J. biol. Chem. 1931. *92*. XXV.

Boothby, W. M., and R. Weiss, Ibidem 1925. *63*. L.

Bürger, M., and H. Kohl, Arch. exp. Path. Pharmak. 1935. *178*. 269.

Carpenter, T. M., Tables, factors and formulas etc., Washington 1939.

Chandler, J. P., and W. H. Chambers, Amer. J. Physiol. 1938. *123*. 34.

Cori, C. F., J. Pharmacol. 1925. *25*. 1.

Cori, C. F., and G. T. Cori, J. biol. Chem. 1928. *76*. 755.

Corkill, B., Biochem. J. 1930. *24*. 779.

Dann, M., and W. H. Chambers, J. biol. Chem. 1930. *89*. 675.

Dickson, B. R., G. S. Eadie, J. J. R. Macleod, and F. R. Pember, Quart. J. exp. Physiol. 1924. *14*. 123.

Dudley, H. W., P. P. Laidlaw, J. W. Trevan, and E. M. Boock, J. Physiol. 1923. *57*. XLVII.

Fischer, S., Arch. Psychiat. 1928. *83*. 205.

—, Z. ges. Neurol. Psychiat. 1933. *147*. 109.

Frank, E., M. Nothmann, and E. Hartmann, Arch. exp. Path. Pharmak. 1928. *127*. 35.

Gabbe, E., Klin. Wschr. 1924. *3*. 612.

Haist, R. E., J. H. Ridout, and C. H. Best, Amer. J. Physiol. 1939. *126*. 518.

Hawley, E. E., and J. R. Murlin, Ibidem. 1925—26. *75*. 107.

Henry, G. W., J. Nerv. Ment. Dis. 1929. *70*. 598.

Himsworth, H. P., J. Physiol. 1934. *81*. 29.

Himsworth, H. P., Lancet 1939. *237*. 1. 65. 118. 171.

Hines, H. M., J. D. Boyd, and C. E. Leese, Amer. J. Physiol. 1929. *88*. 240.

Holten, C., Bibl. Laeger 1929. *121*. 49.

Johansson, J. E., Skand. Arch. Physiol. 1908. *21*. 1.

Johnston, M. W., J. M. Sheldon, and L. H. Newburg, J. Nutrit. 1939. *17*. 213.

Kellaway, C. H., and T. A. Hughes, Brit. med. J. 1923. *1*. 710.

Krogh, A., and P. Brandt Rehberg, Pavlov Jubilee Vol. 1925. 91.

Langfeldt, G., The Endocrine Glands and Autonomic Systems in Dementia Praecox, Oslo 1926.

Lindhard, J., J. Physiol. 1911. *42*. 337.

Lundbæk, K., Undersögelser af Insulinets virkning, specielt paa blodsukkeret og det respiratoriske stofskifte ved kulhydratrig og kulhydratfattig kost. Diss. — Copenhagen, 1943.

LUNDBÆK, K., Acta physiol. scand. 1944, *7*, 18, 29.
LUNDBÆK, K., and G. MAGNUSSEN, Acta med. scand. 1940. *105*. 447.
LUNDSGAARD, E., Upsala Läkaref. förh. 1939. *45*. 143.
LUNDSGAARD, E., N. A. NIELSEN, and S. L. ÖRSKOV, Skand. Arch. Physiol. 1936. *73*. 296.
LUNDSGAARD, E., N. A. NIELSEN, and S. L. ÖRSKOV, Ibidem 1939. *81*. 11.
LYMAN, R. S., E. NICHOLLS, and W. M. S. MCCANN, J. Pharmacol. 1923. *21*. 343.
NIELSEN, N. A., Om Insulinets Virkemaade, Diss. Copenhagen 1932.
REISS, M., and R. WEISS, Z. ges. exp. Med. 1926. *49*. 276.
REITER, G., Z. klin. Med. 1933. *125*. 605.
REITER, P., Nogle Undersögelser over Kulhydratstofskiftet ved Psykoser, Diss. Copenhagen 1925.
SOSKIN, S., Physiol. Rev. 1941. *21*. 140.
SOSKIN, S., and I. A. MIRSKY, Amer. J. Physiol. 1935—36. *114*. 106.
SPENCER, H. J., and W. S. MCCLELLAN, Ibidem 1932. *101*. 95.
SVEINSSON, S. L., Norske Videnskaps-Akademi, I. mat.-naturv. 1941. no. 12.

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On the Effect of Insulin on the Sensitivity of the Respiratory Centre.

By

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Received 12 October 1943.

It is well known that as a rule, in animal experiments as wel as experiments on human subjects, a rise in the ventilation will occur following administration of insulin (DICKSON, EADIE, MACLEOD and PEMBER, 1924; KELLAWAY and HUGHES, 1923; HOLTEN, 1929). The alveolar CO_2 tension does not appear to have been subjected to systematic investigation during insulin action on man, but determinations of the CO_2 content of the blood in animals would seem to indicate that a reduction of this often occurs after administration of insulin (BOOTHBY and WEISS, 1925; CHAMBERS, DEUEL and MILHORAT, 1927). Direct determinations of the sensitivity of the respiratory centre by means of CO_2 respiration do not seem to be available in the literature.

In the sequel we shall give an account of a series of investigations in which the ventilation and the alveolar CO_2 tension were determined after administration of large and small doses of insulin to patients undergoing insulin shock treatment, and it will be shown how on taking these two factors together an idea can be formed of possible changes in the sensitivity of the respiratory centre. The technique in these experiments, the dosage, diet of the subjects, etc. have been described elsewhere (LUNDBÆK, 1944). We shall here merely recall the following facts: The alveolar CO_2 tension was determined in refractory periods according to LINDHARD (1911). All determinations were made in the quiet phase of the insulin action before any hypoglycemic restlessness, spasticity, convulsions etc. had set in. The

experiments lasted for 2—4 hours with determinations every half hour. After large doses of insulin extremely low blood sugar values were obtained.

Ventilation.

The maximal rise in ventilation was determined in 38 experiments on 12 subjects. On a high carbohydrate diet there was an average rise of 1.35 ± 0.23 l per min., on a low carbohydrate diet 1.09 ± 0.23 l per min. In 17 experiments with small doses of insulin a rise in ventilation was seen which averaged 0.32 ± 0.07 l per min. on a high carbohydrate diet, 0.57 ± 0.09 on a low carbohydrate diet.

Alveolar CO_2 Tension.

The maximal change in the alveolar CO_2 tension was determined in 34 experiment on 12 subjects following large doses of insulin. On the high carbohydrate diet there was an average rise of 1.89 ± 0.30 mm, on the low carbohydrate diet the rise averaged 2.08 ± 0.65 mm. In 17 experiments on 7 subjects following small doses of insulin the rise was 1.45 ± 0.30 on a high carbohydrate diet, 2.15 ± 0.70 on a low carbohydrate diet. There is no statistically significant difference between these 4 mean values.

The Sensitivity of the Respiratory Centre.

From the preceding part it will be seen that in general there is a rise in the ventilation as well as in the alveolar CO_2 tension following administration of insulin. If the sensitivity of the respiratory centre is unchanged a rise in the alveolar CO_2 tension should involve a certain rise in the ventilation. The magnitude of this rise cannot be stated with accuracy. It is generally reported that with a rise in the alveolar CO_2 tension of 3 mm, the ventilation rate per minute is redoubled. The relation between the ventilation and the alveolar CO_2 tension, however, differs individually.

It appears from MARIUS NIELSEN's investigations (1936) that in the subjects examined by him there was a correlation between

these two factors varying from 0.24 to 0.71 mm alveolar tension per 1 rise in the ventilation. Previous experimental results on less copious material showed a sensitivity corresponding partly to the highest (CAMPBELL, DOUGLAS and HOBSON, 1914; LILJESTRAND, 1918), partly to the lowest (LINDHARD, 1911) of the values observed by MARIUS NIELSEN. In the present work this relation has not been examined for the individual subjects with CO_2 respiration. Nevertheless it will be possible to form an estimate as to whether any great changes have taken place in the sensitivity of the respiratory centre.

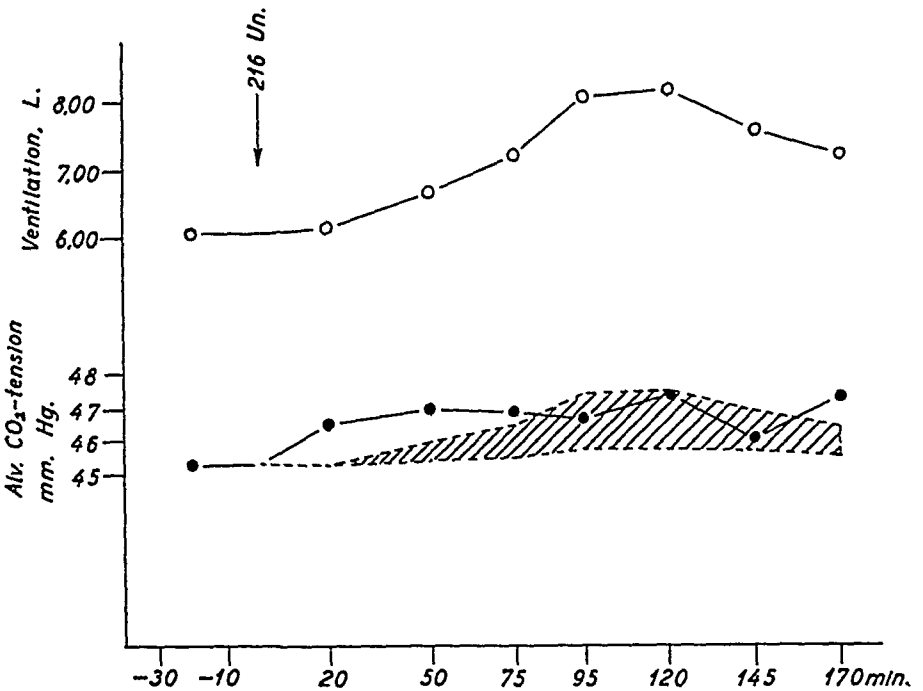
With the material at hand this has been attempted in the following way. On the basis of MARIUS NIELSEN's results such wide limits were chosen as might reasonably be supposed to occur in the normal relation between ventilation and alveolar CO_2 tension. It was assumed, then, that a rise in the ventilation of 1 litre might correspond to a rise in the alveolar CO_2 tension of from 0.2 to 1.0 mm. In diagrams of the ventilation and alveolar CO_2 tension from the individual experiments are marked such values of the alveolar CO_2 tension as would correspond to the extreme values given for a rise in the alveolar CO_2 tension per 1 of the rise in the ventilation. In this way we get a field of varying width within which the values observed of the alveolar CO_2 tension must be supposed to lie, if no change occurred in the sensitivity of the respiratory centre. If the values lie clearly outside this field, a change in the sensitivity of the centre must probably have occurred.

An example of this procedure is shown in fig. 1.

On surveying such a graphic representation of the individual experiments on 11 subjects the following results will emerge.

In the 14 experiments made on them 4 subjects showed distinct lowering of the sensitivity of the respiration centre. In 23 experiments 4 others as a rule but not always showed such a lowering. 1 person exhibited varying conditions, another as a rule a tendency to an increase in sensitivity. Only one subject showed a distinct uniform increase in the sensitivity of the respiration centre in 2 experiments.

Thus even though these results do not give a quite unambiguous picture the conclusion would seem to be warranted that insulin in general has a tendency to lower the sensitivity of the respiratory centre. In some cases it seems, however, that the sensitivity may increase. This corresponds very well to the ge-



Example of graphic estimation of changes in the sensitivity of the respiratory centre.

neral impression gained of the psychic response to the administration of insulin; in most subjects insulin at first produces an increasing dullness and somnolence, but in a few there are at once signs of psychic hyperirritability.

On considering the relation between large and small doses it appears that the reduction in sensitivity did not occur more frequently with large doses than with small doses. The degree of reduction also is not conclusively greater with big doses. This agrees with the fact that dullness and slight clouding of consciousness is one of the first signs of hypoglycemia and so already is present with slight degrees of insulin action. The preceding diet does not seem to be of any significance for the change in the sensitivity of the respiratory centre.

In the above it was taken for granted that a rise in the alveolar CO_2 tension which exceeded what would correspond to the change in the ventilation must be due to a primary accumulation of CO_2 owing to a reduction in the sensitivity of the respiratory centre. There is, however, also the possibility that the increase is secondary in relation to a rise in the alkali reserve ("compen-

sated alkali excess"). Of the factors that might possibly cause a primary rise in the alkali reserve must especially be mentioned the increase of the secretion in the stomach, and the fall in ketones and phosphates in the blood. The two latter factors are, however, of so small an order of magnitude that they will hardly be significant. For the elucidation of the question as to the significance of the secretion in the stomach following administration of large doses of insulin, the alkali reserve was examined in 2 patients, one with a normal secretion of acid, the other with a complete histamin and insulin refractory achylia. Two examinations of each patient brought to light the following facts.

Table 1.

Alveolar CO_2 tension and alkali reserve in a normal and an achylic subject.

		Alv. CO_2 tension (mm Hg)	CO_2 vol% (after saturation at 40 mm)
H. S. (normal) . .	before	41.0	66.1
	90 min. p. inj.	43.8	67.8
	before	38.6	62.2
	90 min. p. inj.	43.5	63.2
E. G. (achylic) . .	before	34.3	58.3
	90 min. p. inj.	36.8	59.3
	240 min. p. inj.	38.3	60.1
	before	36.5	63.7
	90 min. p. inj.	40.7	68.2

It appears from this that in both cases there is an increase in the alkali reserve during insulin action. There is therefore no reason to assume that the observed rise in the alveolar CO_2 tension is secondary to an increase in the alkali reserve conditioned by a "loss of acid" to the stomach. It must be supposed that we have here a primary accumulation of CO_2 with a secondary reparatory rise of the alkali reserve, that is to say, a reduction in the sensitivity of the respiration centre similar to that seen after administration of morphia (LINDHARD, 1911; HIGGINS and MEANS, 1915).

The cause of the difference in the results from animal experiments must be partly found in the fact that the determinations

of the CO_2 tension of the blood in the animal experiments were presumably made at so late a stage of the insulin action that there was greater muscular restlessness, partly possibly in the difference in the response of the various species of animals. Notably it must be pointed out that dogs very quickly seem to show "hyperirritability".

In connection with another examination (LUNDBÆK, 1944) it may be mentioned that a change in the sensitivity of the respiration centre will of course be able to cause a falsification of the RQ. However, the changes observed were as a rule gradual and the respiration before and after the individual experimental period was so uniform that this will probably not give rise to any great errors. The very fact that the sensitivity of the respiration centre is changed will not cause any falsification of the RQ. The error, if any, due to this fact will with the most frequently occurring change, a reduction of the sensitivity, tend to give too low values for the RQ, so that too low an estimate will be made of the rise in the latter.

Summary.

Following the administration of insulin in experiments on animals and human subjects a rise has most frequently been found in the ventilation. In animal experiments and in some few on human subjects a reduction was found in the CO_2 content of the blood.

Determinations of the sensitivity of the respiratory centre during insulin action do not seem to be available.

The results are reported of a series of investigations on the alveolar CO_2 tension in man following administration of large and small doses of insulin on a high and on a low carbohydrate diet. The average rise in the ventilation in these experiments was 1.09—1.35 l per min. after large doses, 0.57—0.32 l per min. after small doses. At the same time the alveolar CO_2 tension rose on the average 1.45—2.15 mm, the rise being the same after large and small doses.

By a graphic representation of the ventilation and the alveolar CO_2 tension and on the basis of previous investigations by MARIUS NIELSEN it can be shown that in nearly all cases there is a reduction in the sensitivity of the respiratory centre after small as well as after large doses of insulin. The preceding diet is of no significance for this change in sensitivity.

References.

Boothby, W. M., and R. Weiss, J. Biol. Chem. 1925. *63*. L.
Campbell, J. M. H., C. G. Douglas, and F. G. Hobson, J. Physiol. 1914. *48*. 303.
Chambers, W. H., H. J. Deuel, and A. T. Milhorat, J. biol. Chem. 1927. *75*. 423.
Dickson, B. R., G. S. Eadie, J. J. R. Macleod, and F. R. Pember, J. exp. Physiol. 1924. *14*. 123.
Higgins, H. L., and J. H. Means, J. Pharmacol. 1915. *7*. 1.
Holten, C., Bibl. Læger 1929. *121*. 49.
Kellaway, C. H., and T. A. Hughes, Brit. med. J. 1923. *1*. 710.
Liljestrand, G., Skand. Arch. Physiol. 1918. *35*. 270.
Lundbæk, K., Acta physiol. scand. 1944, *7*, 1.
Nielsen, M., Untersuchungen über die Atemregulation beim Menschen, Diss. Copenhagen 1936.

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The pH of the Blood during the Action of large Doses of Insulin.

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In a previous paper (LUNDBÆK, 1944) it was shown that very often, after the administration of insulin, a change occurs in the alveolar CO_2 tension, as a rule manifesting itself as a greater rise in the latter than would correspond to the rise in the ventilation. It is probable that we are here concerned with a primary CO_2 excess caused by a lowering of the sensitivity of the respiratory centre.

This finding, however, raises another problem. Does the change in the CO_2 content of the alveolar air, which is identical with that of the blood, cause a change in the acid-base balance of the blood?

A rise in the CO_2 content of the blood will automatically involve a rise in the alkali reserve (chloride shift). This will tend to prevent more considerable changes in the hydrogen ion concentration. Excretion of a more acid urine will act in the same direction, but this mode of regulation will of course function more slowly.

In the aforementioned paper it has already been shown that simultaneously with the rise in the alveolar CO_2 tension, a rise in the alkali reserve may be noted. Whether this is sufficient to maintain the pH of the blood or this cannot be fully maintained, may be settled either by determination of the total and free CO_2 content under the actual CO_2 tension and calculation by Henderson-Hasselbalch's equation, or by direct measurement of the pH of the blood.

In the series of investigations here recorded a direct determination of the pH by means of glass electrodes was chosen. The technique was briefly as follows: the blood is placed under paraffin in a semiglobular vessel. The measuring electrode is immersed in this. The vessel is connected to one of the usual calomel reference electrodes. The measuring is done with a tube potentiometer. Double pH determinations were made on venous blood at the shortest possible intervals before and at various times after administration of large doses of insulin.

The literature only contains a few investigations on the pH of the blood after administration of large doses of insulin. DAY and NIVER (1937) stated that they found a considerable alkalosis after administration of insulin. "Insulin is the best alkalizing agent available". GELLHORN, INGRAHAM and MOLDAWSKY (1938) observing dogs after large doses of insulin found no change in the pH in 6 out of 8 experiments. In one a fall, in another a rise in the pH was observed. Motor unrest, ventilation etc. are not mentioned. BEIGLBÖCK and DUSSIK (1938) almost always find "eine Alkalose, ... wie man sie unseres Wissens sonst nicht zu erzeugen in der Lage ist. Es handelt sich hier also um einen beträchtlichen Eingriff in das sonst so zäh festgehaltene Gleichgewicht der H-Ionenconcentration." This contention is, however, not documented by figures or more detailed statements of the experimental results. It is merely mentioned that the alkalosis may perhaps be due to a blowing off of CO_2. In spite of this it has often been cited. In THOMAS and WILSON's widely circulated report to the Board of Control (1938) in which the physiological basis of the insulin shock treatment is surveyed, this finding is mentioned thus: "Alkalosis, a shift of pH to the alkaline side, is one of the best known changes in hypoglycemia", and it is offered as a possible explanation of the curing of psychotic patients that this alkalosis should in some way or other be able to influence favourably some abnormality in the neutrality regulation of these patients.

Amongst other things because these much quoted investigations were performed by direct determination of the pH of the blood, it was decided to employ a similar method in the series of investigations here reported.

Results.

In all experiments pH was determined before and 70—80 minutes after administration of large coma-inducing doses of in-

sulin, and further in most cases at a later period, 2—4 hours after the injection. At that time the patients were often in coma or precoma.

The fasting values ranged from 7.31 to 7.45. The average of 23 determinations on 6 persons was 7.39. — This corresponds exactly to the result of previous investigations. Examining 196 normal subject ELDAHL (1939) found pH to be 7.29—7.47, averaging 7.39. In 35 determinations on 8 persons FAURBYE (1942) found — also with a glass electrode — pH values from 7.30 to 7.49, averaging 7.42.

In order to determine the significance of an observed change in pH the standard deviation in the double determinations was calculated, $s = \sqrt{\frac{d_1^2 + \ldots\ldots d_n 2}{2\ n}}$. It was found to be equivalent to 1.92 hundredths pH units. The estimation of the mean error in a mean value $\frac{s}{\sqrt{2}}$ is then 1.36 hundredths, and the estimated mean error in the difference between the two mean values is $s\sqrt{1/2 + 1/2} = s$. Since t for 56 observations is 2.00, a difference between two mean values may be said to be significant when it exceeds 1.92×2.00, which means, practically, when it equals or exceeds 4 hundredths pH units.

In the present investigation the changes in pH have on the whole been insignificant. In 13 out of the 23 experiments pH did not change appreciably after the administration of insulin. In 6 experiments a small fall was seen. In two short experiments it amounted to 0.05 pH units, in one long experiment to 0.06 pH units in all.

In 3 experiments a transient fall in the pH amounting to 0.05, 0.04, and 0.05 pH units respectively, was observed about 70 minutes after the injection. In one experiment, a rise of 0.07 was seen in the pH 75 minutes after the injection. — Hence in these 20 experiments no changes were seen in 13 cases, and changes just appreciable with the method employed in 7 cases. In 3 cases, however, greater changes in the pH were observed. In two of them a fall was seen, in both cases in the last period, amounting to 0.13 and 0.11 pH units respectively. In both cases marked spastic unrest of the muscles was noted corresponding to these periods, wherefore the fall in pH must no doubt be ascribed to a rise in the lactic acid of the blood. — In one case

there was a considerable difference in the results of the double determinations in the last period. This might be due to an experimental error, but since there was greatly varying respiration in this period, with alternating hyper- and hypo-ventilation, it seems most probable that the first double determination, which shows a rise of pH amounting to 0.10, was taken during a hyperventilation period.

Summary.

An investigation (with glass electrodes) on the effect of large doses of insulin on the pH of the blood shows that quiet insulin action causes no change, or rarely unsignificant changes, in the pH of the blood. With great muscle unrest or hyperventilation there may, as in subjects not affected by insulin, occur a fall or a rise in the pH. The results obtained decidedly militate against BEIGLBÖCK's contention that there is a regular occurrence of extreme alkalosis after administration of insulin. The most reasonable explanation of his results is that in his experiments there must have been a massive hyperventilation.

References.

BEIGLBÖCK, W., Wien. klin. Wschr. 1938. *51*. 497.
BEIGLBÖCK, W., and T. DUSSIK, Schweiz. Arch. Neurol. Psychiat. 1937. Erg. H. zu *39*. 38.
DAY, G. W., and E. O. NIVER, Texas State J. Med. 1937. *33*. 236.
ELDAHL, A., Nord. Med. 1939. *3*. 2938.
FAURBYE, A., Blodets reaktion ved Epilepsi, Diss. Copenhagen 1942.
GELLHORN, E., R. C. INGRAHAM, and L. MOLDAVSKY, J. Neurophysiol. 1938. *1*. 301.
LUNDBAEK, K., Acta physiol. scand. 1944, *7*, 18.
THOMAS, W. R., and J. G. H. WILSON, Report on cardiazol treatment and on the present application of hypoglycemic shock treatment in schizophrenia, London 1938.

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Fasting Values of Blood Sugar, RQ, and Alveolar CO_2 Tension on High and Low Carbohydrate Diet.

By

KNUD LUNDBÆK.

Received 12 October 1943.

During a series of experiments on the respiratory metabolism etc. after the administration of insulin and glucose (LUNDBÆK, 1944) a number of subjects were kept for some time on a high or a low carbohydrate diet. We shall here give an account of the material, collected on that occasion, of the values of blood sugar, RQ, and alveolar CO_2 tension during fasting on a high and a low carbohydrate diet. (For technique etc., see abovementioned paper.)

The diet was composed by the Hygienic Institute of the University of Copenhagen. It was supplied in weighed portions and the leavings were weighed. The carbohydrate content in the amount of food taken could thus be exactly calculated. The high carbohydrate diet (CH+) contained on the average 512 g daily, the low carbohydrate diet (CH—) on the average 135 g daily. That these diets were not extreme appears from the fact that there was only rarely a trace of acetone in the urine, never acetic acid.

The diet employed was given for a period of at least three days prior to each experiment. The length of this preceding period was chosen in such a way that the effect of the diet on the blood sugar curve after administration of glucose and insulin must be supposed to have attained its maximal level. ADELSBERGER and PORGES (1926) found a characteristic change in the glucose tolerance curve already the next day after a day with a low carbohydrate diet. CHAMBERS (1938) states that the maximal effect of a diet on the glucose tolerance curve is attained in the course of

2—4 days, whereafter it keeps constant for a long time. In these investigations, therefore, 3 days were deemed suitable for the adjustment of a new level of the carbohydrate metabolism. The different intake of carbohydrates on the two diets manifested itself characteristically in the fasting values of the RQ and the alveolar CO_2 tension.

Table 1.

Intake of carbohydrate and fasting values of blood sugar, RQ, and alveolar CO_2 tension.

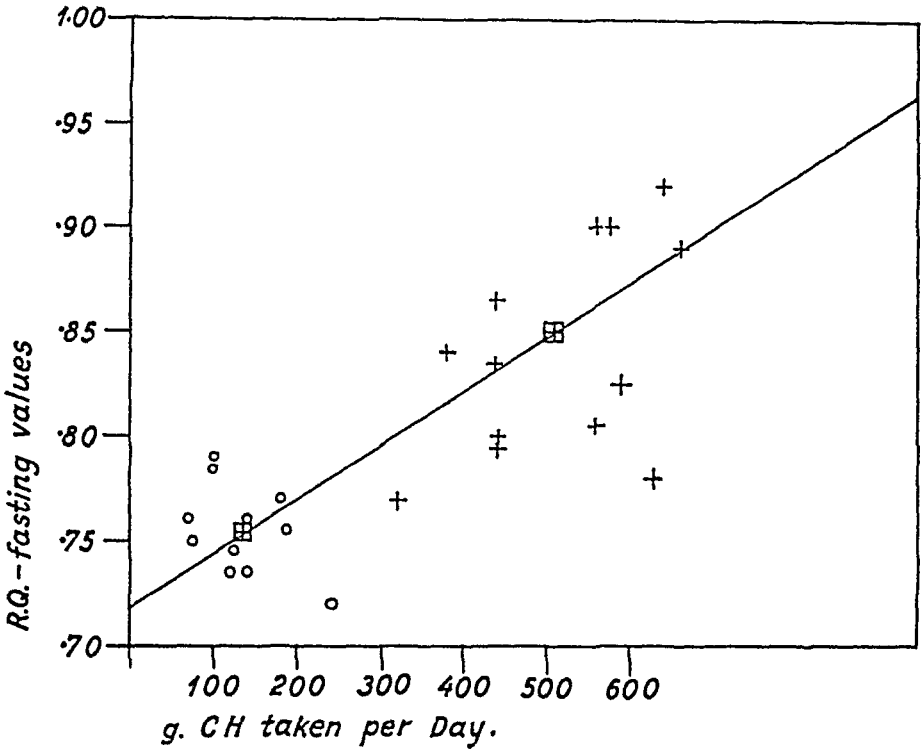
	g. carboh. daily		fasting bl. sug.		fasting RQ		fasting alveol. CO_2 tension mm.	
	CH+	CH—	CH+	CH—	CH+	CH—	CH+	CH—
P. P.	640	143	94	—	0.92	0.76	44.9	—
H. S.	660	188	81	90	0.89	0.77	39.8	38.5
E. G.	378	98	89	85	0.84	0.78	37.9	35.1
H. J.	560	240	83	85	0.90	0.72	43.7	37.7
Ag. J.	435	75	75	74	0.84	0.75	42.2	40.3
I. W.	440	98	85	80	0.87	0.79	42.6	39.1
M. M.	440	—	85	—	0.80	—	38.9	—
J. P.	585	135	91	91	0.82	0.74	40.4	36.7
F. K.	555	191	102	97	0.81	0.75	37.2	34.9
G. P.	318	—	97	—	0.77	—	45.7	—
J. A.	630	70	93	93	0.78	0.76	45.9	43.7
C. C.	574	126	95	92	0.90	0.75	45.6	44.7
An. J.	438	118	88	91	0.80	0.74	42.5	41.3

Table 1 shows the average fasting values of blood sugar, RQ, and alveolar CO_2 tension from the various experiments for each subject separately. It further gives the average intake of carbohydrate per diem for three days preceding each experiment. Hence the individual figures represent average values of several, 3—4, determinations on different days.

The fasting blood sugar does not seem to be affected by the diet. This agrees with previous experiences (MAGNUS-LEVY, 1925), but curiously enough it does not seem to have been systematically investigated before. The total average values are 89 mg % on carbohydrate rich, 88 mg % on carbohydrate poor diet.

For *the fasting RQ values*, on the other hand, a distinct difference is seen on the two diets. On carbohydrate rich diet the average value RQ attained is 0.848, on carbohydrate poor diet 0.755.

In fig. 1 the average RQ values for each subject are given as a function of the amount of carbohydrate consumed for three days preceding each experiment. Though the deviation is considerable



Relation between the carbohydrate content of the diet and the fasting RQ. (+ = carbohydrate rich diet; ○ = carbohydrate poor diet; ⊞ = average value of the two diets.)

a distinct correlation is seen between the carbohydrate taken and the fasting RQ.

The result obtained agrees well with those of earlier investigators. BENEDICT and HIGGINS (1912) determined the RQ after diets with different content of carbohydrate. If the average RQ on the 3rd and 4th days after the transition to the new diet is calculated from their results, it turns out that the RQ on a diet containing 600 g carbohydrate is 0.86, with 400 g carbohydrate it is 0.83, with 200 g carbohydrate 0.79; while with 100 g carbohydrate it is 0.75, and with 0 g carbohydrate 0.72. These results only deviate slightly from the line drawn in fig. 1 which connects the average values of the carbohydrate consumption and the RQ of all the subjects on the two diets here employed (∓).

In KROGH and LINDHARD's investigations (1920) the subjects show an average resting and fasting RQ of 0.90 on a very high, and of 0.75 on a very low carbohydrate diet. The composition of the diet is not given in detail.

CHRISTENSEN and HANSEN (1939) carried out investigations on a high and a low carbohydrate diet, with a carbohydrate content of about 900 g and 50 g respectively. They found average fasting RQ values of 0.93 and 0.74. HANSEN (1942) carried out experiments on the same diets. The average RQ for the various subjects was 0.92 on the diet rich in carbohydrates, and 0.75 on the diet poor in carbohydrates. — Thus it is seen that while the values for the low carbohydrate diet employed by these two authors do not differ essentially from those obtained in the present investigations, the values for the high carbohydrate diet are considerably higher than those here obtained.

This, however, is merely due to the fact that the carbohydrate content in the high carbohydrate diet used by these authors was so much higher than in the one used in the present investigations. According to the line marked in fig. 1 a carbohydrate content of 900 g should give a RQ of 0.95, which corresponds very well with the values given by the above-mentioned authors.

It has previously been shown that the alveolar CO_2 tension is lower on a low than on a high carbohydrate diet (HASSELBALCH, 1912; BENEDICT and JOSLIN 1912; HIGGINS, PEABODY and FITZ, 1916). As will appear from Table I, this is confirmed by the present material. The total average value on carbohydrate rich diet is 42.1 mm, on carbohydrate poor diet 39.2 mm.

Unlike the RQ values, we here see a distinct correlation between the values belonging together, appearing as a parallel shifting in relation to the identity line. This shows that there is an individual fasting CO_2 tension level which varies with the carbohydrate content of the diet.

Summary.

On examining a number of subjects on a high carbohydrate diet (averaging 512 g carbohydrate daily) and on a low carbohydrate diet (averaging 135 g carbohydrate daily) the following results were obtained:

1. Fasting blood sugar was unaffected by the diet, averaging 89 mg % on a high carbohydrate diet, 88 mg % on a low carbohydrate diet.

2. The RQ averaged 0.848 on the high carbohydrate diet, 0.755 on the low carbohydrate. This relation between the carbohydrate content of the diet and the fasting RQ corresponds closely to what can be inferred from the earlier literature.

3. The fasting alveolar CO_2 tension averaged 42.1 mm on a high carbohydrate diet, 39.2 mm on a low carbohydrate diet. It is shown that there is an individual level of the CO_2 tension which varies with the carbohydrate content of the diet.

References.

ABDERHALDEN, E., and E. WERTHEIMER, Pflüg. Arch. ges. Physiol. 1924. *205*. 547.

BENEDICT, F. G., and H. L. HIGGINS, Amer. J. Physiol. 1912. *30*. 217.

BENEDICT, F. G., and E. P. JOSLIN, A study of metabolism in severe diabetes, Washington 1912.

CHAMBERS, W. H., Physiol. Rev. 1938. *18*. 248.

CHRISTENSEN, E. HOHWÜ, and O. HANSEN, Skand. Arch. Physiol. 1939. *81*. 160.

HANSEN, O., Undersögelser over Stofskifteprocesserne ved Muskelarbejde, Diss. Copenhagen 1942.

HASSELBALCH, K. A., Biochem. Z. 1912. *46*. 403.

HIGGINS, H. L., F. W. PEABODY, and R. FITZ, J. med. Res. 1916. *34*. 263.

KROGH, A., and J. LINDHARD, Biochem. J. 1920. *14*. 290.

LUNDBÆK, K., Acta physiol. scand. 1944, *7*, 1.

MAGNUS-LEVY, A., in C. Oppenheimer, Hdb. Biochem. Mensch. Tier. 1925. *8*. 319. 2 ed.

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Experimental Studies on Kidney Function during Sulphate Diuresis.

2. Investigations on the Glomerular Function of Rabbit-Kidneys during Infusion of a Hypertonic Sulphate-Solution.[1]

By

PER SCHOU.

Received 14 October 1943.

In a previous paper (SCHOU, 1943) an account was given of the profuse diuresis which can be produced by means of an intravenous infusion of hypertonic sulphate solution into rabbits, it being possible in this way to raise the excretion of fluid through the kidneys in these animals to 100—150 times the normal diuresis.

In the interpretation of the experiments creatinine clearance was used as an indicator of the ultrafiltration of fluid in the renal glomeruli, and calculations on the motion of the fluid then showed that, if the observed excessive increase in the diuresis is to be explained by the CUSHNY-REHBERG kidney function theory (the filtration — reabsorption theory), it must be based partly on a very considerable reduction in tubular function as regards the reabsorption of fluid, partly on an increase in glomerular filtration, which according to the calculations may amount to more than twice that occurring under normal circumstances.

Is such an increase in filtration possible at all? That is to say, are such physical conditions present in the renal glomeruli that the above-mentioned calculated values for the increased glomerular activity come within the limits of probability?

[1] A contribution towards carrying out this work was granted by The P. Carl Petersen Foundation.

It is this question which will be dealt with below, and our investigation must begin with a brief examination of the forces acting in the Bowmann capsule.

The filtration in the renal glomeruli is supposed to be a purely mechanical process and the factors which determine the extent of the filtration must therefore be *the effective hydrostatic blood pressure in the glomeruli* in connection with *the amount of blood supplied to the glomerular capillaries.*

The first of these

the effective filtration pressure, must, broadly, be the resultant of three partly variable factors, viz. the arterial blood pressure, the decrease in the pressure before the blood reaches the glomerular capillaries, and finally the osmotic pressure of the plasma colloids.

The arterial blood pressure must, in the nature of the case, be of decisive importance for the mechanical filtration process.

The lowest arterial pressure compatible with the excretion of fluid has been found by JANSEN and REIN (1928) to be around 75 mm Hg, just as LASSEN and HUSFELDT (1934) for man found a similar value of about 70 mm. Hg. Other authors (USTIMOWITSCH 1870, GRUTZNER 1875 and STARLING and VERNEY 1925) have previously, in circumstances where the urine contained large amounts of diuretics, found much lower values, around 40 mm. Hg.

While the arterial blood pressure can be measured directly, this is not the case with the lowering of the pressure which occurs on the way down to the glomeruli, so that the statements concerning

the hydrostatic capillary pressure in the glomeruli are based exclusively on indirect determinations.

WINTON (1937) has enumerated the works of earlier authors concerning this factor and its relation to the pressure in the renal vein and ureter, and has then on the basis of his investigations on the pressure conditions in the isolated kidney calculated the hydrostatic pressure in the renal glomeruli and found that it must lie around 2/3 of the simultaneous pressure in the renal artery.

The colloid-osmotic pressure in the glomerular capillaries will reduce the effective filtration pressure to an extent corresponding to the protein content of the plasma, the proteins being assumed to be retained in the capillaries at the ultrafiltration of the other components of the plasma.

According to the statements of most authors the total protein content of the plasma lies around 6—7 %, and the corresponding colloid-osmotic pressure has been found to be about 24 mm. Hg.

The second of the factors determining the extent of the filtration is *the amount of blood flowing through the renal glomeruli*, or in other words, *the rate of blood flow in the kidneys*.

For a long time the investigations of these facts had to be based on direct measurements of the rate of flow from the renal vein and on experiments on isolated organs.

The more recent determinations of the renal blood flow have, however, in the main been made by the flow meter method devised by REIN (1928), which permits the measurement of the rate of blood flow in unopened vessels by a thermoelectric procedure. By means of this technique various authors have investigated the relation between the renal blood flow and the excretion of urine.

JANSSEN and REIN (1928) as well as GLASER, LASZLO and SCHÜRMEYER (1932) found no certain correlation between these factors.

HANDOWSKY and SAMAAN (1935) showed that water diureses were preceded by a progressive rise in the flow in the renal artery.

WALKER, SCHMIDT, ELSOM and JOHNSTON (1937), in experiments on rabbits with water diureses of 0.2—0.5 ml. per min. per kidney, found no constant correlation between the diuresis and the filtration on the one hand, and the renal blood flow on the other hand, even though the variations in these factors might for several periods go in the same direction.

The available literature concerning the physical conditions of the ultrafiltration in the glomeruli does not, however, concern itself with the very profuse diureses which have here been made the subject of investigation. Therefore the questions which are of interest here are still open, viz. first, whether the abovementioned conditions of pressure in the glomeruli may be subject to such large variations that it is possible to explain thereby the values for the filtration calculated in these experiments, and second, whether the kidneys, under such circumstances, are supplied with sufficient blood for a glomerular filtration of the order here calculated to come within the limits of possibility.

As an attempt to answer these questions we shall in the sequel describe a series of experiments in which we have tried to give a collective estimate of the physical forces acting in the glomeruli

under these circumstances, on the basis of investigations of the arterial blood pressure and the renal blood flow, in connection with calculations on the colloid-osmotic pressure of the plasma colloids during sulphate diuresis.

Experimental Technique.

Rabbits weighing 2—3.6 kg. were used for the sulphate diuresis experiments which were carried out according to the method previously indicated by the author (SCHOU 1943). Here therefore we shall merely mention those changes in the technique which the special nature of these experiments necessitated.

The rate of blood flow in the renal vessels was investigated by the flow meter method indicated by REIN (1928) with a modified technique devised by TVEDE-JACOBSEN (1941), since the method will only with this modification allow of measurements at such high inconstant plasma sulphate values as occur in these experiments.

The experiments were performed by means of the flow meter available in the biological laboratory of Medicinalco Ltd. in co-operation with Mr. TVEDE-JACOBSEN, civil engineer, and Mr. V. LARSEN, pharm. cand. The arrangement was such that simultaneously with the flow meter curve the arterial blood pressure was registered, being measured by means of a mercury manometer, while a drop-recorder placed under the bladder cannula likewise electrically registered the amount of the diuresis.

The scale for the minute volume introduced in the graphs below was thus calculated on the basis of the amounts of heat and the temperatures measured.

From the values for the blood flow read directly from the curves the figures for the plasma flow were calculated by means of the hæmatocrite values determined in each separate case, and according to MORISON (1926) and MACCALLUM (1926) these may again be taken as an expression of the amount of plasma flowing through the renal *glomeruli*.

In the first experiments the flow meter cuvette was placed on the left renal vein, and thus the measurement of the rate of blood flow was restricted to one kidney only.

There was, however, a drawback in using the renal vein for the flow meter measurement, in that the rinsing of the vessel often caused contractions in it, so that the vein did not entirely fill the cuvette. Where the contact between the vascular wall and the element was thus defective, the registration entirely failed or at best gave irregular and unreliable curves.

This disadvantage is avoided by placing the cuvette on an artery and in most of the experiments therefore the cuvette was, as recommended by ELSOM, BOTT and WALKER (1937) placed round the aorta after this had been ligated just distally to the left renal artery. Lumbar

branches from the aorta and the superior mesenteric artery were also ligated. Thus the blood flow through both kidneys was measured.

The arterial blood pressure was registered by means of an Hg manometer. Since the circulation of the entire hind part had been put out of function the arterial cannula was placed on the left carotid artery. Blood samples were drawn drop by drop through a T tube on the connecting rubber, so as to avoid a disturbing effect on the blood pressure.

Coagulation of the blood at the inserted cannulas was prevented in all the flow meter experiments by intravenous injection of 5 ctgr. Liquoid Roche. Apparently the animals tolerated this well, but no doubt it was a contributary cause to the fall in the blood pressure which occurred in these experiments.

As previously stated, *the hydrostatic capillary pressure* was calculated to be 67 % of the arterial pressure.

The colloid-osmotic pressure in the arteries was calculated according to the method devised by WIES and PETERS (1937) on the basis of the plasma protein content. This was determined refractometrically with corrections for the constantly varying sulphate content.

As mentioned in a previous paper (SCHOU 1943), *the glomerular filtration* was determined by creatinine clearance. Creatinine analyses were carried out colorimetrically according to the principle of FOLIN with the technique devised by E. NIELSEN (1930).

The hemoglobin content of the plasma was determined colorimetrically a. m. FLEISCHL-MIESCHER.

According to the principles stated in the previous paper (SCHOU, 1943) the renal function is expressed by *the diuresis* and the *glomerular filtration* as also by *the degree of concentration of the urine*, that is, the C index $= \frac{\text{mg. \% creatinine in urine}}{\text{mg. \% creatinine in plasma}}$ and the Excr. %, that is the percentage of the glomerular filtrate which is excreted as urine.

Experimental Results.

The investigations comprise 7 flow meter experiments in which observations were made on the blood flow and pressure during sulphate diuresis, as well as the renal function conditioned by these factors.

As will appear from the following, the arterial blood pressure in the flow meter experiments is somewhat lower than normal; this is due to the incision necessitated by the insertion of the cuvette, probably in connection with the intravenous injection of liquoid "Roche".

Therefore 3 supplementary blood pressure experiments were carried out, in which these possible sources of error are excluded.

As examples we have given below the notes on four of the flow meter-blood pressure experiments (experiments 5, 6, 7, and 8).

Directly photographed flow meter and blood pressure curves are given from 1 of the experiments (5) (Fig. 1).

The blood pressure curve is given from one experiment on blood pressure (8); Fig. 2.

The correlation of the various factors concerning the blood pressure, blood flow, and diuresis in a single experiment (7) is recorded in Fig. 3.

All other experimental results are recorded in Tables I and II.

Notes on Experiment 5. $^{24}/_{5}$ 1940. Male rabbit weighing 3.6 kg. Flow meter experiment.

At 8.15 20 ml. 25 % uretane solution in 0.9 % NaCl solution subcutaneously.
9.15 Operation.
11.15 1.2 gr. creatinine in 20 ml. 0.9 % NaCl solution intravenously, thereafter slow infusion of 0.9 % NaCl solution, 25 ml. in all.
11.49—12.14 Normal period.
12.20—12.36 Infusion of 60 ml. 20 % Na_2SO_4, 10 H_2O.
12.26—12.38 3 sulphate diuresis periods of 4 minutes each.

Notes on Experiment 6. $^{27}/_{5}$ 1940. Male rabbit, 3.6 kg. Flow meter experiment.

At 8.30 22 ml. 25 % uretane solution in 0.9 % NaCl solution subcutaneously.
9.30 1.2 gr. creatinine in 20 ml. 0.9 % NaCl solution subcutaneously.
10.00 Operation. As the cuvette was not in a satisfactory position the abdomen had to be opened a second time, which no doubt caused the considerable fall in the blood pressure that rendered a normal period impossible.
From 12.47—13.01 infusion of 40 ml. 20 % Na_2SO_4, 10 H_2O.
12.53—13.18 4 sulphate diuresis periods of 4 minutes each.

Notes on Experiment 7. $^{29}/_{5}$ 1940. Male rabbit 2.98 kg. Flow meter experiment.

At 8.35 18 ml. 25 % uretane solution in 0.9 % NaCl solution subcutaneously.
10.00 Operation.
11.30 1.2 gr. creatinine in 10 ml. 0.9 % NaCl solution intravenously. Normal period given up owing to absence of diuresis.
From 12.34—12.58 Infusion of 60 ml. 20 % Na_2SO_4, 10 H_2O.
12.38—12.41 1st sulphate diuresis period. Proved a failure.
12.31—13.02 2nd to 8th sulphate diuresis periods.
13.95—13.08 9th sulphate diuresis period.

Notes on Experiment 8. $^{30}/_{5}$ 1940. Male rabbit 2.6 kg. Blood pressure experiment.

At 11.15 Uretane solution in 0.9 % NaCl solution subcutaneously.
12.15 Operation. Blood pressure cannula inserted in this experiment in the left femoral artery.
13.15 1.2 gr. creatinine in 20 ml. 0.9 % NaCl solution subcutaneously.
From 14.51—15.11 Normal period.
15.19—15.34 Infusion of 100 ml. 20 % Na_2SO_4, 10 H_2O. While the blood pressure was continually registered, the diuresis was observed through 9 periods of 2 minutes each, during which, in order to avoid fluctuations in the blood pressure, the blood samples were taken as far as possible continuously, drop by drop.
15.23—15.40 1st—8th sulphate diuresis period.
15.46—15.48 9th sulphate diuresis period.

Table I.

Figures for the observed and calculated factors concerning the excretion of fluid and for the serum-, protein % and hemoglobin % in the 4 experiments, of which the notes are given above while the results are illustrated in Figs. 1—3.

Experiment and Period	Diuresis m/min.	Glomerular filtrate ml/min.	Urine-C. index	Water-Exer. %	Serum-protein %	Hemoglobin %
5 N	0.03	1.3	39.0	2.6	6.7	—
S_1	8.4	16.3	2.0	51	4.5	—
S_2	10.0	14.8	1.5	68	4.3	—
S_3	7.6	13.8	1.8	57	4.4	—
6 S_1	4.7	13.8	3.0	34	3.9	—
S_2	5.8	13.3	2.3	43	3.9	—
S_3	5.1	9.5	1.8	56	4.0	—
S_4	0.5	1.8	3.6	28	3.9	—
7 S_1	3.7	—	—	—	—	—
S_2	6.5	17.8	2.7	37	4.3	—
S_3	6.0	8.4	1.4	72	4.1	—
S_4	7.5	13.0	1.7	58	4.0	—
S_5	6.1	10.3	1.7	59	3.9	—
S_6	4.8	7.9	1.6	61	3.9	—
S_7	3.0	4.9	1.6	61	3.9	—
S_8	1.5	2.9	1.9	52	4.3	—
S_9	0.6	1.5	2.5	40	4.6	—
8 N	0.1	7.1	81.0	1.3	5.7	98
S_1	7.0	20.5	2.9	34	4.2	82
S_2	10.5	16.5	1.6	64	4.2	62
S_3	9.8	13.9	1.4	70	3.0	58
S_4	10.3	14.4	1.4	71	2.9	57
S_5	7.8	10.7	1.4	72	2.8	54
S_6	3.8	5.6	1.5	67	2.9	56
S_7	2.6	4.7	1.8	56	3.1	60
S_8	1.7	3.4	2.0	50	3.5	63
S_9	1.7	4.7	2.9	35	4.4	82

Table I shows that the kidney function, after the intravenous infusion of sulphate, follows the lines mentioned in the previous paper. There is a pronounced increase in the excretion of urine which, according to the calculations in conformity with the filtration-reabsorption theory, must be caused partly by increased glomerular filtration, partly by a reduction of the tubular reabsorption, expressed by the low values for the C. index.

Curve 1 shows that as the basis for the calculated increase in glomerular function may be observed a rise in the renal blood flow and also in the arterial blood pressure.

This rise occurs immediately at the beginning of the infusion and is followed after a few minutes by the sudden setting in of the diuresis.

The renal blood flow: Before the injection of sulphate the flow meter curves, in two vein experiments in which the registrations were confined to one of the kidneys, showed figures for the renal blood flow around 12—15 ml. per min; while the arterial experiments, totalling 5, where the blood flow of both kidneys was registered, showed values of 25—32 ml. per min. A single

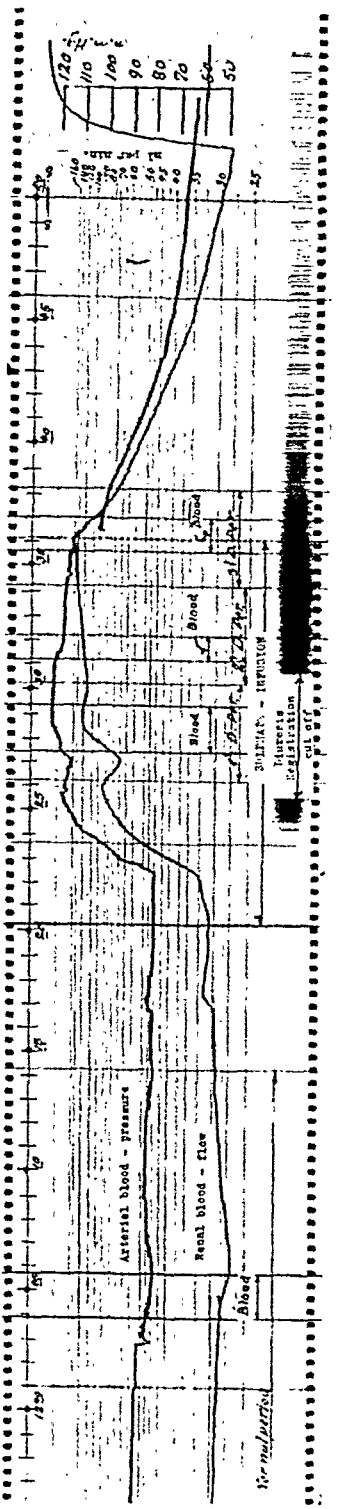

Fig. 1. The renal blood flow for both kidneys is directly registered, expressed in ml per minute. The arterial blood pressure is likewise registered directly, expressed in mm. Hg. Each of the small vertical lines below the curves registers the excretion of a drop of urine, the closeness of the lines expressing the magnitude of the diuresis. This registration failed for a moment in the middle of the experiment. The blood pressure is shown by the upper curve.

experiment, the one here illustrated, presents as low a value as 12 ml. per minute.

Calculated per gr. kidney weight this gives a blood flow of about 1.5 ml. per min., 0.7 in a single experiment (6).

These figures are lower than the average blood flow of 3.2 ml. per min. for the rabbit's kidney found by WALKER, SCHMIDT, ELSOM, and JOHNSTON (1937).

After the sulphate infusion has set in, the flow meter curves show a distinct increase in the renal blood flow, for now 2—3 times as much blood passes through the kidneys as before. In a single experiment (Exp. 6) the blood flow is even increased 4—5 times.

Blood pressure. The rise in the blood pressure curves is as a rule greatest at the beginning of the infusion period, after which it is replaced by a slow fall, which is continued after the infusion has ceased, while the rise in the blood flow sets in somewhat more gradually and is then also at a certain moment, at latest at the end of the infusion, replaced by a fall of the curve.

Upon closer consideration of the curves it will be noted that, apart from the above-mentioned difference in the times for the maximal increase in blood pressure and blood flow, there is a marked tendency for the two curves to run parallel, so that even quite small fluctuations in the blood pressure curve manifest themselves simultaneously in the blood flow curve. This was even the case in those experiments where the curve indicated the blood flow through a vein, a fact which besides showing an anticipated correlation between blood pressure and blood flow may at any rate be taken as a sign of a confidence-inspiring precision in the two mutually independent modes of registration.

As previously mentioned, the rise in the blood pressure in these flow meter experiments starts from very low initial values, about 50—70 mm. Hg.

The increase amounts to 35—55 mm. Hg., so that the blood pressure values attained in this way are approximately on a level with the normal blood pressure of the animal.

In the supplementary blood pressure experiments, 8—10 inclusive, in which the incision necessary for the determination of the blood flow has been omitted, there is also a transient rise in the arterial blood pressure as a consequence of the sulphate infusion. In this case it amounts to 10—25 mm. Hg. and is thus somewhat lower when the initial values are near the normal than at the lower initial values in the blood flow experiments.

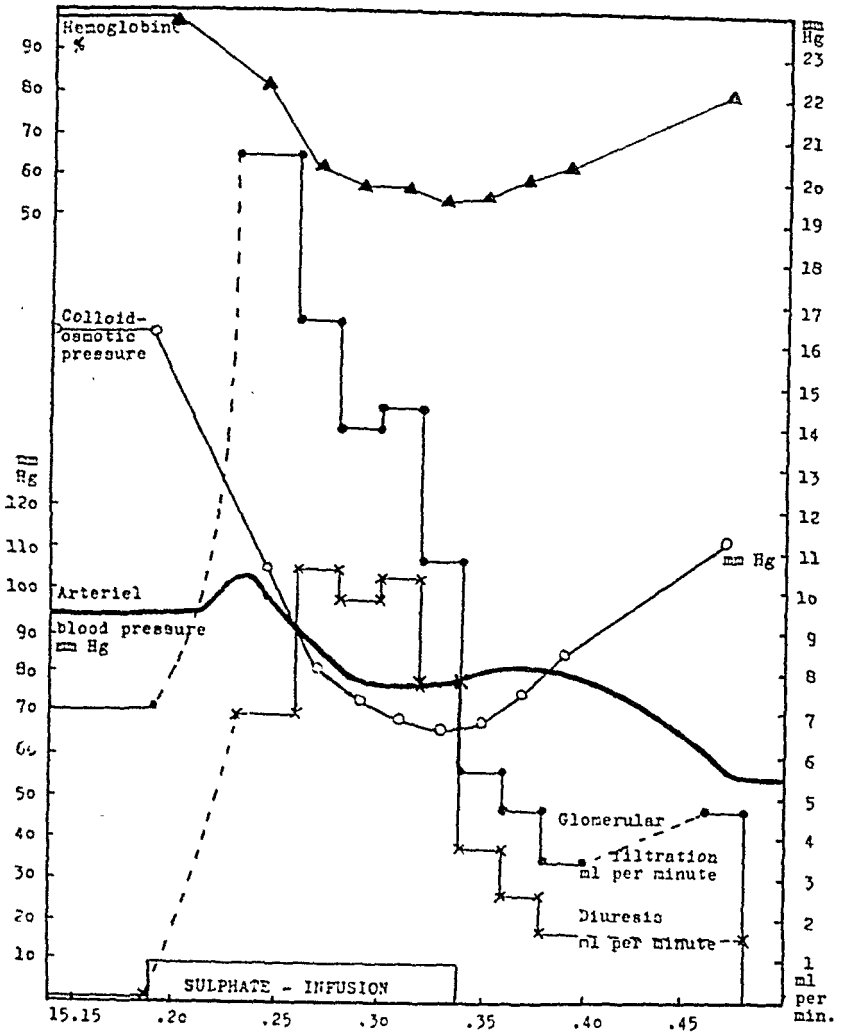

Fig. 2. Experiment 8. Diagram of the relation between
the arterial blood pressure ——
the colloid-osmotic pressure of the plasma colloids ○ ○ ○
the hemoglobin % ▲—▲—▲ ▲
the diuresis ×---×---× ×
and the glomerular filtration ● -●-●-●

From figs. 2 and 3 it will appear that the rise in the arterial blood pressure is accompanied by a simultaneous fall in *the colloidosmotic pressure of the serum proteins* as a result of the dilution of the serum caused by the infusion of fluid.

The serum protein content in the normal periods, before the sulphate infusion, was found to range from 5.5 to 7 %. These figures, however, cannot be regarded as any fully valid expression of the normal protein content in the serum of rabbits, for when

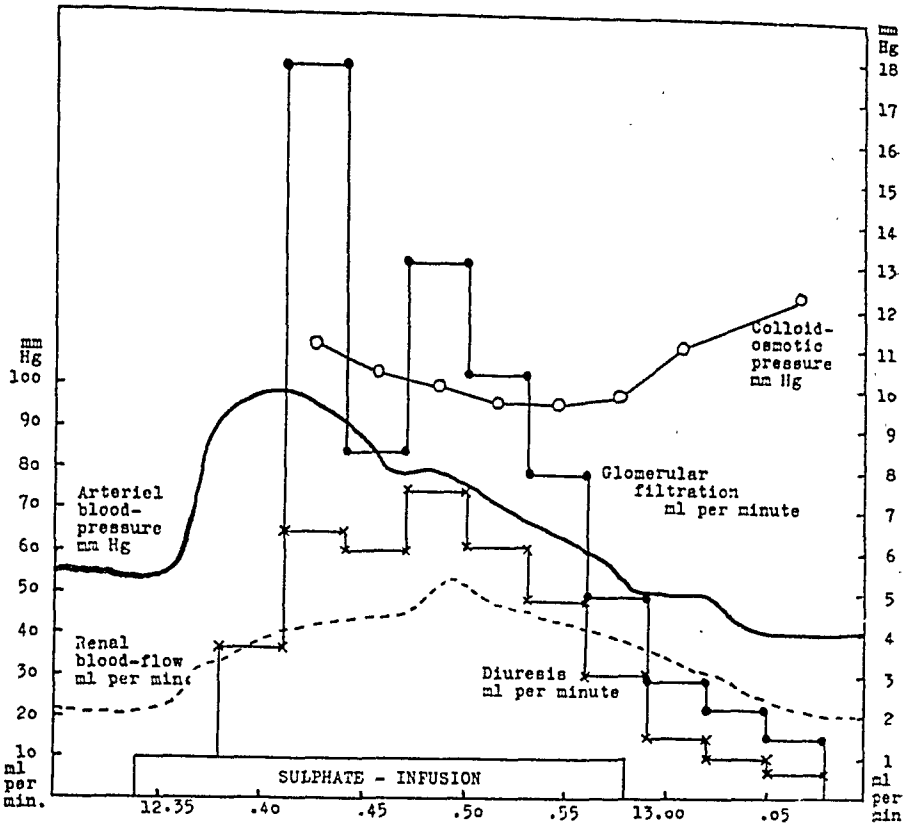

Fig. 3. Experiment 7. Diagram of the relation between
the arterial blood pressure ——————
the colloid-osmotic pressure of the plasma colloids ○—○- -○
the renal blood flow - - - - - -
the diuresis ×—×—×
and the glomerular filtration ●—●—●—●

The determination of the filtration was unsuccessful in the first sulphate diuresis period of this experiment.

these blood samples were taken small quantities of NaCl solution had in most cases been infused.

After the rapid sulphate infusion has started the protein content, as well as *the hemoglobin percentage*, drops to 75—50 % of the original values; it differs somewhat in the various experiments, depending on the amount of infused liquid per unit of time, the size of the animal, and the diuresis.

The importance in this respect of the excreted amount of fluid may be estimated by the rise in the serum protein and hemoglobin percentages towards the end of the experiments, a shortly after the profuse diuresis has set in.

Discussion.

On the basis of the experimental results, the correlation between the renal blood pressure and the renal blood supply on the one hand, and the glomerular filtration and the amount of fluid excreted on the other hand, may be discussed.

As will appear from Figs. 3 and 4, the rather constant low C. index found in the sulphate diuresis experiments in the previous work involves a marked tendency in these experiments also to a proportionality between the glomerular filtration and the diuresis, so that our statements concerning variations in the filtration rate will also in the main apply to the amount of fluid excreted.

If the curves and tables are compared the direct correlation in the individual experiments between the blood pressure on the one hand and the filtration and diuresis on the other hand is plainly seen.

The same applies, though hardly with such regularity, to the relation between the renal blood flow and the glomerular filtration.

This is best seen in experiments where the conditions have been observed continuously during several short periods. Thus Fig. 2 (Exp. 8) shows the dependence of the glomerular function on the blood pressure, while Fig. 3 (Exp. 7) gives a picture of the relation between the blood pressure, renal blood flow, glomerular filtration, and diuresis (unfortunately the determination of the filtration was unsuccessful in the 1st period of this experiment).

Hence it turns out that the increase in glomerular filtration, which is one factor in the greater augmentation of the diuresis immediately after the infusion of the sulphate solution in the blood stream, is favoured simultaneously by a rise in the arterial blood pressure, an increase in the renal blood flow, and a reduction of the resistance originating from the plasma colloids.

In the last stages of the sulphate diuresis the order of magnitude of the filtered amount of fluid often lies below the normal values found before the beginning of the experiment (Fig. 2), and this reduction of the glomerular function runs parallel with a fall in the arterial blood pressure, decreasing blood flow (Fig. 3), and rising colloid-osmotic pressure in the serum.

That the diuresis can, under these circumstances, still be as

high as about 10—20—30 times the normal values, must therefore be ascribed to the tubular reabsorption which remains reduced, the C. index keeping fairly unchanged around 2 during the whole of the sulphate diuresis.

Now, in order to find out the physical conditions in the glomeruli for the calculated augmented filtration we must consider the *absolute* values for the renal plasma flow in the observed diuresis periods, and for the *effective* filtration pressure in the renal capillaries within the same periods.

The numerical values for *the renal plasma flow* may, as previously stated, be calculated on the basis of the readings for the renal blood flow by means of the plasma %.

The values thus calculated for all the experiments are shown in Table II.

On comparing the figures for the different experiments presented in this table it must be remembered with respect to the values for blood flow and filtration that in experiments 1, 2, and 3 these only apply to one kidney, while in the determination of these quantities in experiments 4, 5, 6, and 7 they include both kidneys. In order to facilitate the comparison double values are given in parenthesis in the unilateral experiments.

In those experiments in which the blood flow has been measured on the renal vein (1, 2, and 3) the amount of fluid excreted must be added to the reading in order to obtain the actual measure for the quantity of blood supplied. Hence in these experiments the values for the blood flow are given as the sum of the reading and the diuresis per minute.

As will appear from the table, the blood supply to the renal glomeruli has been found to be very variable from one animal to another in these experiments.

With respect to this factor the various experiments have presented values ranging from 20 to 130 ml per minute.

This fact appears with special distinctness in experiments 5 and 6 here quoted. In these experiments the animals were of equal size but the renal blood flow was 4 times larger in one than in the other.

This fact is in accordance with the results of MAYRS and WATT (1922) as well as the investigations of WALKER, SCHMIDT, ELSOM, and IOHNSTON (1937).

The problem which is of special interest, however, is whether the amount of blood which is at any time supplied to the renal

Table II.

The observed and calculated values for the physical factors which must be assumed to underlie the ultrafiltration in the glomeruli in the 7 flow meter and blood pressure experiments given here.

Experiment and Period	Renal blood-flow ml per min.	Renal plasma-flow ml per min.	Glomerular filtrate ml per min.	Filtration %	Arterial blood-pressure mm Hg.	Glomerular blood-pressure mm Hg.	Arterial coll. osm. pressure mm Hg.	Effective glomerular coll. osm. pressure mm Hg.	Resultant effective filtration-pressure mm Hg.
1 S_1	32 (64)	23 (46)	5.2 (10.4)	23	65	43	9	11	32
2 S_1	25 (50)	19 (38)	1.2 (2.4)	6	92	61	7	7	54
S_2	20 (40)	13 (26)	0.8 (1.6)	6	60	40	11	12	28
3 N	15 (30)	8 (16)	0.8 (1.6)	10	60	40	16	17	23
S_1	36 (72)	28 (56)	5.4 (10.9)	19	74	49	8	9	40
S_2	24 (48)	20 (40)	3.5 (7.0)	18	60	40	7	8	32
4 N	28	17	0.5	29	60	40	16	17	23
S_1	50	38	11.5	30	105	70	9	11	59
S_2	75	59	10.1	17	100	67	8	9	58
5 N	30	15	1.3	9	90	60	21	22	38
S_1	100	67	16.3	24	115	77	12	14	63
S_2	130	87	14.3	17	118	79	11	12	67
S_3	100	66	13.3	20	108	72	11	13	59
6 S_1	32	24	13.8	58	100	67	10	16	51
S_2	31	23	13.3	58	85	57	10	16	41
S_3	28	19	9.5	50	70	47	13	20	27
S_4	13	10	1.8	18	45	30	10	11	19
7 S_2	42	27	17.8	66	95	64	11	22	42
S_3	43	29	8.4	29	82	55	10	12	43
S_4	50	35	13.0	42	77	51	10	14	37
S_5	48	34	10.3	30	71	47	10	12	35
S_6	43	31	7.9	25	65	43	10	12	31
S_7	40	29	4.9	17	55	37	10	11	26
S_8	35	25	2.9	12	46	31	11	11	20

glomeruli is *sufficient* to render possible the calculated rise in the filtration.

This question is answered by the values for *the filtration percentage* given in Table II, that is to say, the percentage of the plasma flowing through the glomerular capillaries which is ultrafiltrated in the glomeruli. This is highly variable in the different experimental periods — ranging from 6 to 66. The mean value — in so far as we can speak of a mean value where the margin of variation is so wide — lies at 26 %. WALKER, SMITH, ELSOM, and JOHN-

STON (1937), who are the only investigators who have previously made determinations of this factor on rabbits by flow meter experiments found as the mean of 29 determinations a percentage of 30 (ranging from 18 to 50).

The above-mentioned variations in the filtration percentage seem to run fairly parallel with the figures for the glomerular filtration but *as the chief result with respect to the blood flow it may be established that the figures for the filtration percentage have not in any case in these experiments been found to be so high as to express an impossible or merely an improbable relation between the amounts of fluid filtered and supplied respectively.*

In the calculation of the other factor, *the effective filtration pressure in the glomeruli*, as the difference between the effective glomerular pressure and the colloid-osmotic pressure of the serum proteins, the numerical values will be rather uncertain, but if, as indicated by WINTON (1937), the fall in pressure from the arteries to the renal glomeruli is put at 33 % of the arterial pressure, and the effective resistance caused by the plasma colloids in the glomerular capillaries is calculated from the protein content in the arterial blood corrected by means of the filtration percentage, then there is a possibility of arriving at some idea of the order of magnitude of the effective filtration pressure.

Table II shows this factor calculated in the above-mentioned way in our 7 experiments.

As an example may be given the facts in the first sulphate diuresis period (S_1) in Exp. 5. The renal blood flow is 100 ml per min., the plasma percentage is 67, which results in a renal plasma flow of 67 ml. per min.

Since the filtration in this period is 16.3 ml. per min. the filtration percentage will be 24.

The arterial blood pressure shows a slightly falling tendency in the course of the period. The mean value may be put at 115 mm. Hg. which, when we have reached the glomeruli, has been reduced to 77 mm. Hg.

The colloid-osmotic pressure in the arterial blood has been found to be 12 mm. but owing to the concentration of the plasma in the glomeruli caused by the filtration the colloid resistance at the conclusion of the ultrafiltration must be $12 \times \frac{100}{100 - 24} =$ $= 16$ mm. Hg. As the effective resistance during the whole filtration period must be selected a mean value of 14 mm. Hg.

The resulting effective filtration pressure in the glomeruli will thus be about 63 mm Hg.

A perusal of the last column in the table shows that the effective filtration pressure in the glomeruli thus calculated with a few exceptions amounts to about half the arterial pressure.

The same ratio has been found for the frog by HAYMAN Jr. (1929).

This will mean, then, that the lowest value observed for this factor compatible with glomerular filtration in these experiments lies around 20 mm Hg, corresponding to an arterial pressure of c. 40 mm and *this agrees with the statements of previous authors* (USTIMOWITSCH 1870, GRUTZNER 1875, STARLING and VERNEY 1925) *concerning the lowest arterial pressure compatible with diuresis.*

The conclusion to be drawn from these experimental results will thus be that both for the blood flow in the renal glomeruli and for the filtration pressure a very wide margin of variation has been demonstrated.

The calculated values of the various factors that condition the glomerular filtration have turned out to be of such an order of magnitude that they are fully compatibly with the great increase found in the glomerular ultrafiltration, if it be assumed that the values for the creatinine clearance are a measure of the amount of the ultrafiltration.

At the same time one of the conditions is now present which will enable us to explain the sulphate diuresis in accordance with the filtration-reabsorption theory.

A following paper shall deal with the investigations on the tubular function of the kidneys during sulphate diuresis.

Summary.

In continuation of previous investigations, in which it was shown that the excessive increase in diuresis after intravenous infusion of a hypertonic sulphate solution in the blood stream of the rabbit can be explained by a combination of an increased filtration of fluid in the renal glomeruli and a reduced reabsorption of fluid in the tubules, the glomerular function has been investigated in the present paper.

1) In flow meter experiments it has been possible to demonstrate, simultaneously with the greatly increased excretion of fluid, a distinct increase in the renal blood flow. The figures for

the renal blood flow were found to lie between 30 and 130 ml per min. for both kidneys, so that the margin of variation is wide.

2) The arterial blood pressure, too, showed a transient rise of 35—55 mm Hg. The rise was fairly quickly superseded by a fall to values lying below the normal blood pressure of the animal.

3) The calculated approximate values for the colloid-osmotic pressure of the plasma colloids at first showed a falling tendency as a consequence of the large infusion of fluid, later again a rising tendency owing to the loss of fluid by diuresis.

4) A final calculation shows that the physical factors which condition the glomerular filtration are of such an order that the increase in the ultrafiltration calculated on the basis of the creatinine clearance determinations becomes possible.

References.

BAINBRIDGE, F. A., and C. L. EVANS, J. Physiol. 1914. *48*. 278.

ELSOM, K. A., PH. A. BOTT, and H. SCHÜRMEYER, Arch. exp. Path. Pharmak. 193. *167*. 292.

HANDOWSKY, H., and A. SAMAAN, J. Physiol. 1935. *83*. 30 P.

HAYMAN, J. M. Jr., Amer. J. Physiol. 1927. *79*. 389.

JANSSEN, H., and H. REIN, Arch. exp. Path. Pharmak. 1928. *128*. 107.

LASSEN, H. C. A., and E. HUSFELDT, J. clin. Invest. 1934. *XIII*. *2*. 263.

MAC CALLUM, D. B., Amer. J. Anat. 1926. *38*. 153.

MAYRS, E. B., and J. M. WATT, J. Physiol. 1922. *56*. 120.

MORISON, D. M., Amer. J. Anat. 1926. *37*. 53.

NIELSEN, E., Bibl. Laeger 1933. *125*. 461.

REIN, H., Z. Biol. 1928. *87*. 394.

SCHOU, P., Experimentelle Undersögelser over Sulfatdiuresen, Diss., Köpenhamn 1942.

—, Acta Physiol. Scand. 1943. *6*. 79.

STARLING, E. H., and E. B. VERNEY, Proc. Roy. Soc. Lond. 1925. B. *97*. 321.

TVEDE-JACOBSEN, I. K., Acta Physiol. Scand. 1941. *2*. 249.

WALKER, A. M., C. P. SCHMIDT, K. A. ELSOM, and C. G. JOHNSTON, Amer. J. Physiol. 1937. *118*. 95.

WIES, C. H., and J. P. Peters, J. clin. Invest. 1937. *16*. 93.

WINTON, F. R., Physiol. Rev. 1937. *17*. 408.

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On the Alleged Relationship between the Erythrocyte Membrane and the Fibrinogen.

By

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Received 3 November 1943.

Nothing is known regarding the fate of the erythrocyte membrane after hemolysis of the erythrocytes. The ghosts are disintegrated and disappear from the blood stream. Evidently, they are broken down by enzymes like other blood constituents.

Some authors have discussed the possibility of a relationship between the stroma protein and the fibrinogen, assuming a more or less direct transformation of the stroma protein into fibrinogen. REYMANN in 1924, after reviewing the literature and referring to his own abundant experimental material, stated that the formation of fibrinogen and globulins in the plasma of horses during diphtheria- and staphylolysinimmunization closely follows the diminution of hemoglobin as the anemia develops under the influence of the toxins. He concluded that the main protein of the erythrocytes, the globin, was transformed into plasma proteins, into fibrinogen and globulins.

This theory was followed up and further developed by DAVIDE in 1925. He found that antisera against fibrinogen from the guinea-pig, the dog, and to a certain extent also from man, showed a resemblance to hemolytic antisera causing a very strong hemolyzing effect and producing a hemolytic anemia in animals. This he considered to be due to some antigenic relationship between the fibrinogen and the erythrocyte stroma, which is known to possess the antigenic properties of the erythrocytes giving rise to

hemolysins. He mentioned, however, that horse fibrinogen was very poor in this respect, and that sheep fibrinogen was devoid of antigenicity. Nevertheless, he concluded not only that there is an antigenic resemblance between the fibrinogen and the stroma protein but also that the stromata, after hemolysis, are converted into fibrinogen. The difficulty of preparing fibrinogen completely free of stromata, could possibly explain some of his experimental results.

In a recent paper by FÅHRAEUS, FAGERBERG and FAGERBERG (1941) particular attention was paid to the question of hemolysis of the erythrocytes and the increase in the fibrinogen content following an intensified hemolysis. Irrespective of whether the hemolysis was caused through the intravenous injection of 40 ml of distilled water in a rabbit or through the injection of lysolecithin, there was a rapid increase in the fibrinogen content of the plasma, closely following the decrease in the number of erythrocytes. This can be best demonstrated through one of their diagrams.

The close connection between the destruction of the erythrocytes and the increase in the fibrinogen content of the plasma, as found by REYMANN, is clearly demonstrated. The authors concluded only, that there is some genetic relationship between the two processes, without making any statement regarding a possible transformation of the erythrocytes into plasma proteins.

The results of FÅHRAEUS and his coworkers, however, allow more far-reaching conclusions to be drawn. It is evident from their experiments that the increase in the fibrinogen content of the plasma can not be due to a transformation of stroma protein into fibrinogen, since the stroma protein of the hemolyzed erythrocytes makes up in weight only about one-sixth of the amount of fibrinogen formed.

During the course of $2^3/_4$ days the erythrocyte count in the rabbit dropped from 5.4 to 4.3 millions, thus a reduction of 20.5 per cent in the cell volume or 6 per cent of the blood volume with a normal hematocrite value of 30. Counting with 30 per cent dry substance in the erythrocytes the loss will be 1.8 g in 100 ml of blood. The stroma protein makes up only 4 per cent of the dry substance of the cells, thus 72 mg of stroma protein in 100 ml of blood, which recalculated on plasma makes 102 mg or a loss of stroma protein amounting to 1.02 per thousand. The rise in fibrinogen was 8.3—2.6 = 5.7 per thousand. In another experiment, where hemolysis was produced by injecting distilled water, the

loss of stroma protein during 6—7 days was 0.7 per thousand calculated on plasma, whereas the rise in fibrinogen was 4.8 per thousand.

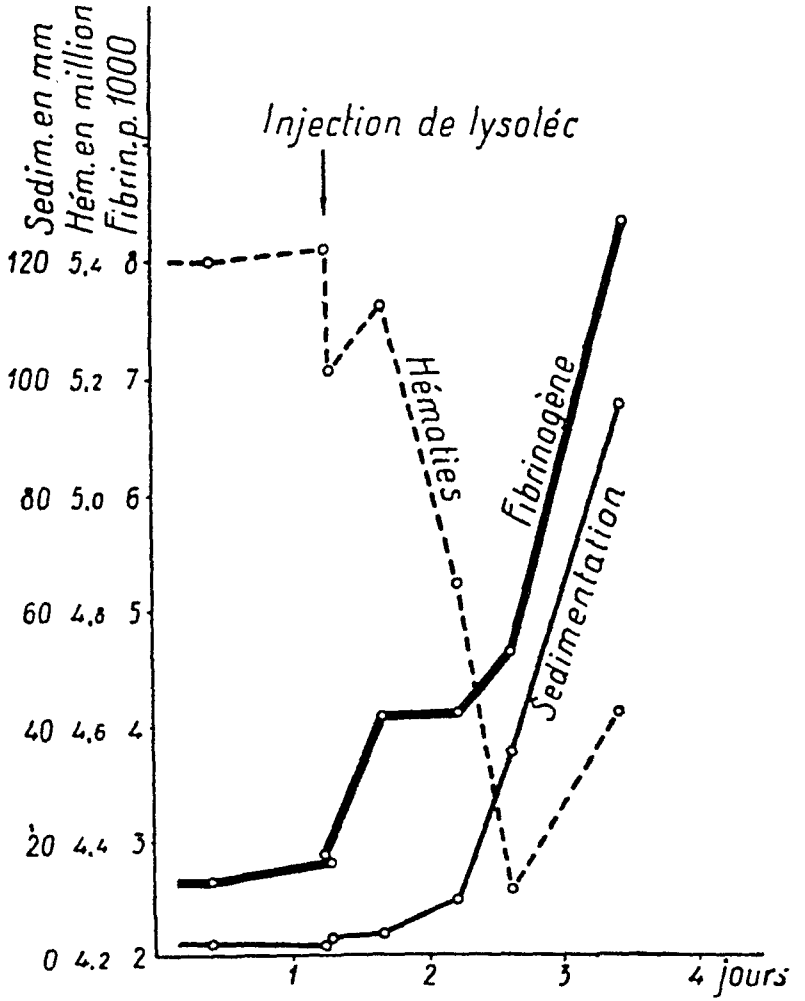

Figure I. The influence of the intravenous injection of lysolecithin in a rabbit on the erythrocyte number, the fibrinogen content of the plasma and the sedimentation rate. (FÅHRAEUS, FAGERBERG and FAGERBERG 1941.)

The short period of time, 3—6 days, during which these changes occur does not allow of any considerable destruction of newly regenerated blood corpuscles; hence the possible contribution to the fibrinogen formation given by the stroma protein must be negligible.

There is another aspect not considered by previous authors which is very important, and which makes any discussion on a

direct transformation of the cell protein into plasma proteins superfluous. As has been known for a long time, the different blood proteins have their own amino acid content (see LUSTIG and HAAS 1930). Each of them contains a specific number of the various amino acids arranged in a certain structural pattern. The present author (1932) found this to be the case also with the stroma protein, named stromatin. Table I demonstrates the different compositions of some blood proteins as indicated by their different amino acid contents.

Table I.

Amino acid content of some blood proteins (Jorpes 1932).

	Arginine Per cent	Histidine Per cent	Tyrosine Per cent	Tryptophane Per cent
Stroma protein	5.80	2.63	3.01	1.46
Fibrin	7.97	2.23	5.54	4.19
Serum globulin	5.82	2.07	5.18	2.21
Hemoglobin	3.32	7.70	2.84	1.26
	3.32 (VICKERY 1928)	7.64 (Vickery 1928 and 1942)		

Even if the stroma protein, also called stroma fibrin, shows a superficial resemblance to fibrin, forming long-chained fibers, it has an amino acid content of its own.

Consequently, the stroma protein as well as the other blood proteins arise through specific synthethic procedures, the raw material probably being single amino acids. These figures show that there can hardly be any discussion about a transformation of hemoglobin, for example, or the stroma protein, into fibrinogen. The synthesis of the globulins as well as of the fibrinogen in the liver runs its own course even if it is greatly stimulated through all kinds of foreign influences upon the liver. Such an influence is exerted through an excessive destruction of the erythrocytes.

Summary.

The author calls attention to a previously unrecognized circumstance with regard to the composition of the stroma protein of the erythrocytes and of the fibrinogen, which allows the conclusion to be drawn that the stroma protein, after hemolysis, can not be transformed into fibrinogen. As a matter of fact, the amino acid

content of the different blood proteins is quite different, each one having its own specific composition. It is evident, therefore, that *there is no direct transformation of stroma protein into fibrinogen* when the erythrocytes disintegrate and the ghosts disappear from the blood stream. Furthermore the author, interpreting recent experimental findings of FÅHRAEUS et al., calls attention to the fact that the amount of fibrinogen produced during the course of a few days through the influence of the hemolyzed blood corpuscles exceeds sixfold the amount of stroma protein liberated through the hemolysis.

References.

DAVIDE, H., Acta Med. Scand. 1925, Suppl. XIII.
FÅHRAEUS, R., S. E. FAGERBERG and E. FAGERBERG, Ibidem, 1941. *108.* 1.
JORPES, E., Biochem. J. 1932. *26.* 1488.
LUSTIG, B., and P. HAAS, Biochem. Z. 1930. *231.* 472.
REYMANN, G. C., Z. Immunitätsforsch. 1924. *41.* 209, 265, 284.
VICKERY, H. B., and C. S. LEAVENWORTH, J. Biol. Chem. 1928. *79.* 377.
—, Ibidem. 1942. *144.* 719.

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Hemolysis by Hypertonic Solutions of Neutral Salts.

By

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Received 4 November 1943.

The fact that erythrocytes undergo hemolysis not only in hypotonic, but also in extremely hypertonic solutions of neutral salts was known even in the days of HAMBURGER, but, while the phenomenon of hemolysis in a hypotonic environment has long been the subject of exhaustive study both from the clinical and the experimental points of view, little attention has been paid to hemolysis by concentrated solutions. Practically nothing has been known regarding its possible significance in clinical practice.

The primary object of the investigation to be reported here was to gain some conception of possible divergences in the resistance of the red blood corpuscles to hypertonic saline solutions, in connection with different illnesses. During the course of these studies, however, my interest was aroused in the theory regarding hemolysis in concentrated solutions which will occupy the major part of the present paper.

To begin with, it may be mentioned as an illustration that human erythrocytes suspended in a 5 M NaCl solution undergo complete hemolysis within two to five minutes. At lower concentrations, a greater or a lesser proportion of the total amount of erythrocytes becomes hemolyzed at a slower rate, the rate being slower, and the degree of hemolysis lower, in proportion to the weakness of the concentration of the sodium chloride. In solution with a lower than normal concentration there is, as a rule, hardly a trace of hemolysis even after the lapse of twentyfour hours. After about three hours, there is a relative stagnation in the

hemolytic process in all concentrations, and the degree of hemolysis then remains fairly constant. In this investigation, therefore, the value after three hours was used throughout as a measure of the hemolysis effect in concentrated solutions. After this time, NaCl causes complete hemolysis at a concentration of about 4.5 M, and beginning hemolysis at about 1.25 M. (See table I.)

I. Observations and Discussion Concerning the Mechanism Involved in Hemolysis by Hypertonic Solutions.

A good explanation of hemolysis by hypotonic solutions was early provided by the observation that the cell membrane of erythrocytes is permeable to water but not to most of the substances dispersed in water. The reason why neutral salts were also unable to penetrate the membrane was believed to lie in the fact that the membrane prevents the passage of the cations. If the osmotic pressure in the external medium be made lower than that prevailing inside the membrane this will cause a preponderance of the pressure of the enclosed electrolytes. Equilibrium can only be reached by the penetration of water into the erythrocytes, which thereby increase in volume. Hemolysis occurs when the membranes, which are stretched as a result of the increase in volume, finally burst (to use a popular expression).

It was a logical consequence for HAMBURGER, and other investigators who have made a study of this mechanism, to assume that hemolysis by concentrated solutions is due to the working of the same forces. According to their view, the high osmotic pressure in the surrounding fluid must obviously compress and dehydrate the erythrocytes, this being reflected in a decrease in the volume. A limit to this reduction in volume, however, would be set by the restricted amounts of erythrocyte water which could be set free, and when this limit is reached the erythrocyte membranes would literally be burst by the external pressure and the hemoglobin thus liberated.

It is my belief that the simplest evidence against this theoretical conception is to be found in the following experiments, using solutions of substances which readily penetrate the erythrocyte membrane.

As is well known, urea and the NH_4 salts are substances possessing this property. In pure solutions of this type of substance, at all concentrations, red blood corpuscles must more or less

rapidly undergo hemolysis analogous to that caused by hypotonic solutions. The dissolved substance diffuses into the erythrocyte at the same concentration as that present in the external fluid, and the latter can then not achieve any counter-pressure to the electrolytes enclosed in the erythrocytes and which cannot pass through the membrane. This hemolysis can be prevented by dissolving in the outer fluid a substance such as NaCl, which will not penetrate the membrane, to make an isotonic concentration. From the point of view of osmosis, a solution of this type will always be isotonic with the erythrocyte, irrespective of the concentration of the substance passing through the membrane.

If hemolysis by hypertonic solutions were a process of an osmotic nature one naturally ought not to obtain hemolysis in such a system even with high concentrations of the permeating substance.

In the experiments I have made, using solutions of, among other substances, urea and NH_4Cl, to which NaCl had been added to make isotonia, it was found, however, that urea caused hemolysis at approximately the same osmotic pressure as corresponding hypertonic NaCl solutions, and NH_4Cl with a somewhat lower molar concentration (approximately equal to LiCl). When different halogen salts of NH_4 were used it was found that these were hemolytically active in the same order as the corresponding Na salts viz. $J' > Br' > Cl'$.

Thus, substances which readily penetrate the cell membrane cause, at high concentrations, a hemolysis which seems to be fully analogous to that occurring in solutions of (according to the current conception) non-permeant substances. The membrane barrier to cations — which constitutes the essential condition for the acceptance of the "osmotic" hemolysis theory — appears to play no part in the process of hemolysis by hypertonic media.

In order to investigate whether this membrane barrier is *in any way* a factor to be reckoned with when the external ionic pressure is as high as it was in these experiments, I endeavoured to titrate the Cl content in the unhemolyzed corpuscles which had been suspended for three hours in concentrated solutions of LiCl, NaCl, KCl, and RbCl. My method was to centrifuge the suspended erythrocytes and carry out the titration on the centrifugate after hemolysis by distilled water. (It would, of course, have been better to titrate direct on the cation. However, as this procedure involves technical difficulties, and as the cations can only enter the erythro-

cyte in conjunction with the Cl ions, I considered the titration on the chloride to be justified.) It was observed that after three hours there was still a considerable difference between the Cl′ concentrations in the external fluid and in the erythrocytes, when the initial concentration of the former was as low as 1 M. When a suspension fluid was used with the initial concentration of 2.5 M the Cl concentration in the erythrocytes after three hours was about the same as in the surrounding fluid and in some cases (KCl and NaCl) even appreciably higher (see table III). In other words, it seemed as though the cell membrane in the latter case had been fully permeable to the salts used.

That this elimination of the barrier function of the membrane was relative, not absolute (as a result of injury to the cell membrane), is proved by the following observation. If erythrocytes which have been suspended, for example, for one hour in 3 M NaCl are transferred to a physiologic saline solution they hemolyze instantaneously. The same phenomenon is observed if they are changed over into somewhat stronger NaCl solutions of a concentration of up to 1 M. A beautiful example of this phenomenon is provided by $MgCl_2$ solutions of concentrations over 4 M, in which even a very slight diminution of the concentration in the external medium gives immediate hemolysis. In my opinion, the only feasible interpretation of these findings is that the theoretically hypertonic or isotonic solutions having a lower concentration than the starting solution mentioned above have become hypertonic for the erythrocytes, which are now highly saline, and which therefore undergo in these solutions an osmotic, *paradoxical "hypotonic" hemolysis.* For such a theory to be acceptable, however, the membrane's barrier function must still exist and constitute a relative obstacle to the diffusion outward, at least at a sufficient speed, of the ions inside the membrane. With permeant substances of the urea type, this "paradoxical" hemolysis does not, of course, occur.

As early as 1922, Acél and Lorber made another observation which points to the possibility that hemolysis by hypertonic solutions may not be a process of a simple osmotic nature. They demonstrated that different neutral salts do not produce hemolysis in *equivalent* concentrations but rather with extremely varying osmotic pressures. Later investigators (Akagi 1930, Jodlbauer, 1935) reached approximately the same conclusion, apparently being unaware of the work of Acél and Lorber.

According to the findings of these authors, the anion plays the chief part in bringing about the hemolysis effect of a salt, in concentrated solutions. ACÉL and LORBER noted that, in equivalent solutions of Na salts, the anions, as far as hypertonic hemolysis is concerned, can be grouped into the series $SCN' > J' > Br' > NO'_3 > Cl' > SO_4''$. AKAGI and JODLBAUER observed the same order in respect of the halogenides (JODLBAUER's findings applied to both K and Na salts). But the cations also showed divergences among themselves. JODLBAUER constructed the series $Li \cdot > Cs \cdot > Rb \cdot > Na \cdot > K \cdot$ with respect to the univalent alkali cations. In this case, however, it was a question of differences of considerably lower magnitude. JODLBAUER used the chlorides for his investigation.

In my own investigations I have arrived at the same order among the anions (see tables I—II). As will be seen, the differences are considerable. Molar solutions of KSCN, NaSCN, and NH_4SCN all hemolyzed completely after three hours, and among the SO_4 salts I only obtained hemolysis with $(NH_4)_2SO_4$, and even with a 5 M solution it was not complete. Possibly Li_2SO_4 might also cause hemolysis in hypertonic solutions, but the precipitate which forms in connection with the use of this salt makes it difficult to carry out determinations. The order between the (halogen-) anions was the same irrespective of whether Na, K, NH_4, Rb, Ca, or Mg salts were used.

The differences between the effects of the univalent alkali cations were relatively small, and the order was obviously not constant in respect of different anions. With chlorides I noted the grouping $Li \cdot > Na \cdot > K \cdot > Rb \cdot$, and with iodides $Rb \cdot > K \cdot > Li \cdot > Na \cdot$.

Table I.

Percentage of hemolysed erythrocytes after 3 hours in solutions of certain neutral salts (0.5—5.0 M).

	$CaCl_2$	$MgCl_2$	NH_4Cl	LiCl	NaCl	KCl	RbCl	NaBr	NaJ	NaSCN
0.5 M.	0	0	0	0	0	0	0	0	—	—
0.75 M	24	5	0	0	0	0	0	0	0	10
1.0 M.	52	18	5	5	0	0	0	4	16	50
1.25 M	—	—	—	—	5	—	—	16	78	100
1.5 M.	100	54	10	12	7	0	0	18	100	100
2.0 M.	100	100	12	12	13	7	4	23	100	100
2.5 M.	100	100	35	30	24	16	10	50	100	100
3.0 M.	100	100	62	56	38	31	30	66	100	100
3.5 M.	100	100	90	85	55	—	38	79	100	100
4.0 M.	100	100	100	100	76	—	58	100	100	100
4.5 M.	100	100	100	100	100	—	86	100	100	100
5.0 M.	100	100	100	100	100	—	100	—	—	—

Table II.

Percentage of hemolysed erythrocytes after 3 hours in solutions of alkali-iodides (0.4—1.6 M).

	NaJ	LiJ	KJ	RbJ
0.4 M	0	0	0	0
0.6 M	0	0	0	28
0.8 M	0	10	18	100
1.0 M	16	60	100	100
1.2 M	54	100	100	100
1.4 M	87	100	100	100
1.6 M	100	100	100	100

The bivalent ions in the Ca group gave a much greater hypertonic hemolysis effect, although the differences between the various elements in this group also were slight. However, I believed I could distinguish a series $Ca^{\cdot\cdot} > Mg^{\cdot\cdot} > Sr^{\cdot\cdot} > Ba^{\cdot\cdot}$ (the order between the last two being uncertain).

Table III.

Molar concentration of Cl′ in suspension fluid and erythrocytes. The estimation was made, 3 hours after human erythrocytes had been suspended in 1 M and 2.5 M solutions of LiCl, NaCl, KCl and RbCl.

A = molar concentration of Cl′ in the outer solution.
B = molar concentration of Cl′ in the erythrocytes.

	LiCl		NaCl		KCl		RbCl	
	A	B	A	B	A	B	A	B
1 M	0.90	0.51	0.88	0.57	0.87	0.57	0.92	0.47
2.5 M	2.24	2.11	2.23	2.57	2.11	2.82	2.31	2.49

$MgCl_2$ exhibited a strange feature, in that the hemolysis did not appear most rapidly in the highest concentrations. While a 3 M $MgCl_2$ solution, for example, gave almost instantaneous hemolysis, the reaction proceeded much more slowly in higher concentrations, and in a 5 M solution it was not even complete after three hours. Precipitates which had appeared by this time (in all probability in the hemoglobin) might cause one to suspect that the delayed hemolysis was only a seeming one, and that the remaining turbidity was due to these precipitates. A microscopic examination carried out after about ten minutes, when the hemolysis in a 3 M solution was complete, revealed, however, that the erythrocytes in a 5 M solution were practically unaltered, and did not even display the changes in form generally occurring in hypertonic solutions. That the red cells in all probability were really fairly intact both from the physical and the chemical standpoints was also proved by the fact that a rapid diminution of the concentration of the external medium from 5 M to 4 M, for instance, caused instantaneous

hemolysis without any residual precipitate (as an expression of the phenomenon of paradoxical hypotonic hemolysis discussed earlier in this paper). In other words, there would seem to be a true inhibition of hypertonic hemolysis in concentrations of $MgCl_2$ over 3 M. I can only place this observation on record here, and make no attempt to explain it. The same phenomenon occurs, in a less pronounced form, with $CaCl_2$, but not with $MgBr_2$ or $CaBr_2$.

For the rest, one has the definite impression that fundamentally different mechanisms may be the cause of hypertonic hemolysis in respect of different salts. SCN, J, Ca, and NH_4 salts, for instance, cause colour changes in the hemoglobin which are without doubt a reflection of a chemical alteration in the molecule. To what extent this feature is of significance as an explanation of the phenomenon of hemolysis by hypertonic solutions must be left open to discussion.

ACÉL and LORBER (1924) were the first to point out the striking conformity between the ion series just described and the so-called Hofmeister series relative to, among other features, the swelling-promoting action of neutral salines on certain colloidal systems. As a consequence of this observation, they believed they were justified in concluding that hypertonic hemolysis must come about as a result of a saline action on colloids forming part of the erythrocytes. Most of the later investigators who have studied this question (PENATI and DONATI, 1938, JODLBAUER, 1935, and others) have come to the same conclusion. In all probability also, there are good reasons for believing that changes in the degree of hydrophilia of the erythrocytic colloids may be the explanation of hypertonic hemolysis.

With this theory as a starting point one might assume that the reaction in question could be brought about in one of two different ways, viz. through a lesion in the *membrane* by which the hemoglobin could flow out into the solution in which the corpuscles are suspended, or through swelling of the *hemoglobin* which thereby would become capable of bursting the membrane. (There is also a third possibility, namely, that the hemoglobin molecule might become changed in such a way that the colouring constituent could penetrate out through the undamaged membrane.)

JODLBAUER has made an observation which is direct evidence in favour of the first alternative. If NaCl, for example, be added in increasing amounts to suspended erythrocytes hemolyzed by pure water, the previously clear solution first becomes opaque, i. e. stromata reappear (a phenomenon sometimes wrongly termed reversible hemolysis). When the concentration of NaCl is again

increased, the stromata disappear once more and the solution becomes clear at approximately the same concentration of saline at which hypertonic hemolysis is usually produced. I myself have also observed this feature, and I was able to establish that this hemolysis is not "reversible". When the concentration of saline is again decreased the stromata of the corpuscles cannot be made to reappear. I have also noted that it is always very difficult to find any stromata in specimens in which hypertonic hemolysis has occurred. These findings would indicate that an actual dissolution of the stromata takes place in hypertonic saline solutions.

JODLBAUER does not discuss this phenomenon in any detail. He makes one reference to BECHHOLD, who considered that from the theoretical standpoint saline solutions causing salting out of the membrane colloids but not of the hemoglobin ought to be able to cause hypertonic hemolysis. In my opinion, such a theory is not capable of explaining the observation described above. If it were a question of a salting out of the stroma colloids, the stromata ought rather to be more plainly visible in hemolyzing saline concentrations than to disappear as was described here.

As the most commonly known effect of strong salt solutions is their power to precipitate (salt out) colloids from their disperse phase, the idea of a dissolution of the stroma colloids under the conditions in question here may seem unattractive. In reality, however, similar phenomena have been observed in several other connections. Even fairly stable systems of colloids which are not readily dissolved can be made to go into solution precisely by using concentrated solutions of neutral salts. With such solutions, VON WEIMARN (1926—1927) for example, was able to bring about dissolution, even at room temperature, of substances such as silk and cellulose which are otherwise difficult to dissolve. It is of interest to note that the most active substances in his tests were found to be salts of the LiJ and $Ca(SCN)_2$, type, i. e. combinations of ions which must be regarded as extremely active even when it is a question of hypertonic hemolysis. In my own experiments it was noted, for instance, that CaJ_2 caused complete hemolysis almost instantaneously even in an osmotically isotonic concentration, and in all probability $Ca(SCN_2)$ (which I did not have an opportunity to test) would be still more hemolytically active.

The possibility that swelling (increased hydration) of the hemoglobin is the explanation of hypertonic hemolysis is at present nothing more than a hypothesis. Theoretically speaking, however, it is not beyond the bounds of possibility. TAKEI (1921), and later DONATI and PENATI (1938), have shown in connection with hematocrite tests that the red cell volume, with increasing concentrations of NaCl in the surrounding medium, decreases at first (a

feature to be expected from the standpoint of the osmotic theory), but that after reaching a minimum value at approximately four times isotonia it again increases, attaining a level close to the starting values in pre-hemolytic concentrations. This phenomenon may, however, have some connection with the increasing amount of saline penetrating into the erythrocyte, and in all probability it can not be arbitrarily interpreted as swelling of the hemoglobin.

In this connection the microscopic appearance of the process of hemolysis by hypertonic solutions is almost puzzling. I attempted to study the effect of high concentrations of saline, from the morphologic standpoint, by placing a drop of 5 N NaCl on the edge of a "native" sample (a sample of capillary blood without an anticoagulant, using a coverglass and an object glass). When the hypertonic solution streams in, the erythrocytes assume to begin with the typical flattened, abruptly folded (never "crenated") shapes which in all probability are evidence of the diminution in volume seen in connection with osmotic dehydration, and which also appear in the hematocrite experiments mentioned in a preceding paragraph. Just before the hemolysis begins, however, the corpuscle suddenly begins to swell (the swelling often being localized, at first, at some part of the edge of the cell body), and very rapidly becomes spherical. The hemolysis then takes place. The whole procedure, with its extremely rapid swelling, is strongly reminiscent, in fact, of the picture seen in hemolysis with distilled water, and it is difficult to believe that it can be due to a successive increase in the hydration of the colloids. In any case, it is here a question of a genuine swelling easy to distinguish from the formation of spherical "microcytes" often seen in isotonic solutions when the corpuscle comes into contact with the surface of the glass.

In other words, it looks as though hemolysis by hypertonic solutions were a rather complex process. In any event, it seems fairly certain that neutral salt, in sufficiently concentrated solutions, passes rather rapidly into the erythrocyte at the same concentration as that in the suspension fluid, the resistance constituted by the membrane's impermeability to the cations thus being overcome, although this obstructing function is not eliminated irreversibly. A gradual dissolution of the stroma colloids seems to take place, but it looks as if it were not this phenomenon but rather a swelling of the contents of the erythrocyte that is the direct cause of the type of hemolysis under discussion.

II. Clinical Observations.

The only investigation known to the writer which touches upon the subject of the possible clinical significance of hypertonic hemolysis has been carried out by PENATI and DONATI. They did not study the actual phenomenon of hemolysis, however; their attention was directed rather towards the changes in the red cell volume occurring in pre-hemolytic concentrations. Their clinical material included normal subjects, cases of pernicious anemia and simple hypochromic anemia, and of familial acholuric jaundice (hemolytic jaundice) and cirrhosis hepatis. They observed only very slight variations in the appearance of the volume curves in the different illnesses. A feature of some interest in connection with my results is that the swelling phase preceding the hemolysis in the curve relative to the hypochromic anemias showed a tendency towards higher concentrations when compared with the curves obtained from other illness groups.

I myself have investigated the hypertonic hemolysis effect in concentrated NaCl solutions, using not only normal subjects but also a non-selective clinical series of 150 patients with different illnesses from the medical wards, and finally a selected series comprising cases of hemolytic jaundice, pernicious anemia, acute hepatitis, polycythemia, and hypochromic anemias of diverse origin.

The following is a description of my general method.

I used for my investigation citrated venous blood in concordance with the WESTERGREN method for the erythrocyte sedimentation reaction. Twenty cmm. of whole blood were added to 4 ml. of NaCl solution. After this suspension had been standing for three hours at room temperature the unhemolyzed erythrocytes were centrifuged down, the supernatant fluid pipetted off, and the hemoglobin in this fluid transformed into reduced hemoglobin with sodium hydrosulfite after it had first been alkalized with $NaHCO_3$. The extinction value for this solution was read off in a Pulfrich stufenphotometer with a violet S_{66} filter. The values found were expressed in per cent of a control solution with complete hemolysis in 0.4 per cent H_3N *from the same sample of blood.* (A calibration curve determined empirically had to be used.)

The results in control tests using erythrocytes washed with physiologic NaCl, and blood without anticoagulant (capillary blood) did not deviate in essentials from those obtained by the above-mentioned technique.

For the tests in which the hypertonic hemolysis effect in connection with different salts was studied, certain alterations had to be made in

the technique. With some salines (e. g. the Li salts) which give precipitation with sodium hydrosulfite, the hemoglobin determinations were made without reducing the oxyhemoglobin. In a few solutions (RbCl in concentrations over 3 M, and KBr over 3 M) the specific gravity of the erythrocytes even after 3 hours remained lower than that of the solution, and the cells could therefore not be thrown down by centrifugation. In these instances the degree of hemolysis was determined by measuring the extinction values for the dispersion of the remaining erythrocytes with a red S_{50} filter, as compared with a sample containing suspensions of unhemolyzed red corpuscles. The last-named method in particular implies a considerable likelihood of error in the form of the altered relative indices of refraction in erythrocytes at high saline concentrations and these values can therefore not be regarded as having any great degree of accuracy.

By means of an examination of ten healthy normal subjects the values relative to hypertonic hemolysis with NaCl, shown in table I, was determined. Similar complete hemolysis curves were also plotted from a few of the pathologic cases, two patients being taken as a rule from each illness group.

In order that the work of estimation in connection with the clinical material should be as simple and methodical as possible, the degree of hemolysis with NaCl, at concentrations of 2.5 N and 4 N after three hours, was determined in respect of thirty normal subjects. With these concentrations it was found to be 24 ± 6.3 per cent and 76 ± 8.7 per cent, respectively. The degrees of hemolysis at these two concentrations were taken as being representative of the appearance of the hemolysis curve, and they were used as a basis in estimating the material.

With regard to the result of the clinical investigation I need say little, since in most of the illnesses examined the hypertonic hemolysis values were within the normal variation limits. The only exceptions were found in connection with a group of anemias with a very low colour index. The following features of interest may be mentioned.

1. In 5 cases of familial hemolytic jaundice (of the classical type) in which the erythrocytic resistance to hypotonic solutions was definitely lowered, the resistance to hypertonic saline solutions was normal.

2. In 12 cases of pernicious anemia, in the untreated stage, the values were arbitrarily distributed within the normal limits. In 3 instances the resistance was studied four times during the course of the treatment without any alteration in the values being observed.

3. In 3 cases of polycythemia vera, 11 of acute hepatitis, 4 of cirrhosis hepatis, and 8 of obstructive jaundice the values were also normal.

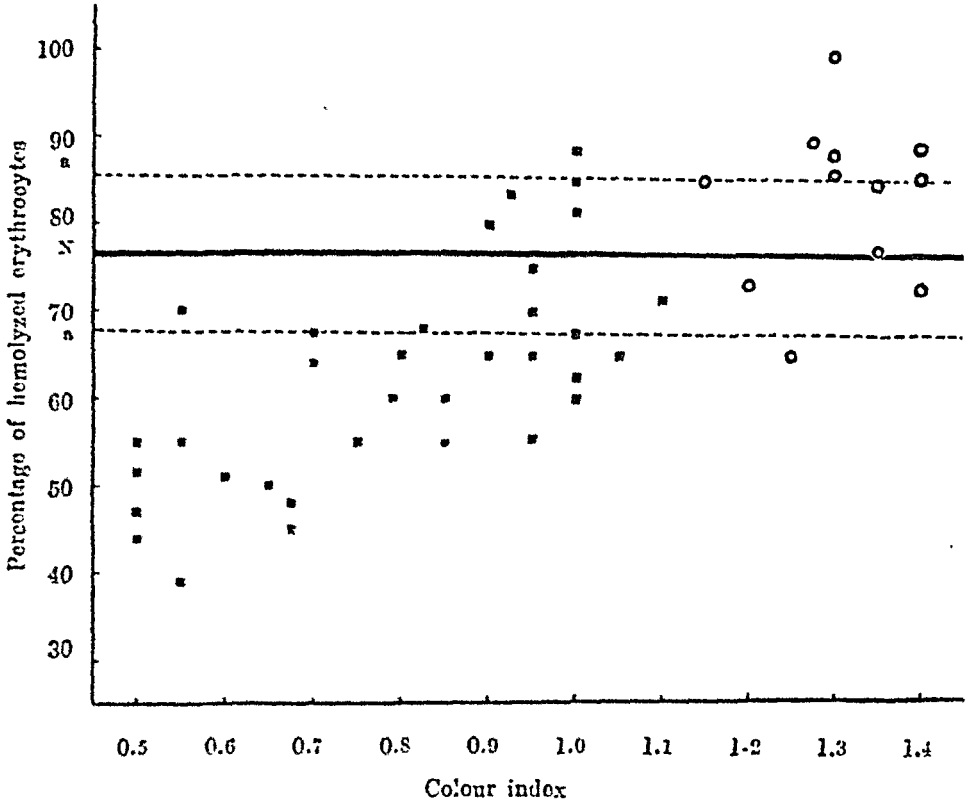

Fig. 1. Degree of hemolysis in a 4 M solution of NaCl after 3 hours in 34 cases of hypochromic-normochromic anemias, and 12 cases of pernicious anemia. The cases are distributed with respect to colours index. A correlation exists at least for index values below 1. No correlation whatever was found to the red cell count or hemoglobin percentage. N indicates the medium level of hemolysis (at the levels of standard deviation) in 30 normal cases. ○ Pernicious anemia. ■ Other anemias.

4. The only category in which noticeable divergences from the normal values could be established was the group of hypochromic anemias, which for my purpose embraced all the cases with a low colour index, e. g. anemia sideropenica simplex, and anemia due to cancer, infection, or hemorrhage. In these instances the resistance was on the whole higher than normal, this feature being most apparent in the values from the 4 M NaCl concentration. From figure 1 it will be seen that the resistance to hypertonic NaCl solutions seems to increase with a falling colour index. Otherwise, there were no signs of relationship with any definite illness. Of the 5 cases showing the highest resistance (lower than 50 per cent hemolysis with a concentration of 4 N NaCl), 3 had anemia sideropenica simplex, 1 carcinosis, and 1 Osler's disease with protracted intestinal hemorrhage.

It seems within reason to endeavour to link this feature up with a possible genuine decrease in the relative hemoglobin content of the erythrocytes, in these hypochromic anemias. In view of the uncertainty still prevailing regarding the factors essential to the origin of hypertonic hemolysis, however, it is perhaps wisest to refrain for the present from attempts at an explanation.

Summary.

An investigation on hemolysis by hypertonic solutions of neutral salts has proved that the phenomenon occurs without any regard to the so-called impermeability of the red cell membrane to cations. With concentrations in which hemolysis is produced, alkali chlorides, at least, penetrate almost in their entirety into the cell interior within a short space of time.

In this connection a description is given of a phenomenon not previously reported, which has been termed paradoxical hypotonic hemolysis.

Earlier observations concerning the significance of the ion series in the processes of hemolysis discussed in this paper have been confirmed in the main.

In a clinical investigation, the erythrocytes were found to possess normal resistance to hypertonic solutions of NaCl in a number of diseases of interest, among others familial hemolytic jaundice. Divergences from the normal values were only noted in connection with hypochromic anemias of diverse origin, in which the red blood corpuscles showed a raised resistance to hypertonic solutions of NaCl.

References.

Acél, D. and L. Lorber, Biochem. Z. 1924. *147*. 557.
Akagi, S., Mitt. Med. Akad. Kioto 1930. *6*. 35.
Ege, R., Biochem. Z. 1922. *130*. 99.
—, Ibidem 1923. *134*. 234.
Jodlbauer, A., Arch. exp. Path. Pharmak. 1935. *178*. 719.
Meyer, K., and M. Dunkel, Zeitschr. f. physik. Chemie, Bodensteinfestband 1931.
Pauli, Wo., and E. Valko, Kolloidchemie der Eiweisskörper, Dresden 1933.
Penati, A., and G. Donati, Arch. Sci. med. 1938. *65*. 759.
Ponder, E., and G. Saslow, J. Physiol. 1930. *70*. 161.
Ponder, E., and G. Saslow, Ibidem 1931. *73*. 267.
Takei, T., Biochem. Z. 1921. *123*. 104.
Weimarn, P. P. v., Kolloidzschr. 1926—27. *40—41*. 120.
—, Ibidem 1927. *42—43*. 43.

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Glycerol Oxydation in the Animal Organism.

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There seems to have been little research into the question of the metabolism of glycerol, probably because of the lack of suitable methods of analysis. As I have devised a method of glycerol analysis for another purpose (1943), a method capable of application to series determinations on blood, it was so to say the obvious course to make use of it in an investigation into glycerol metabolism.

The method is based on an extremely sensitive colour reaction, which is specific for glycerol and is brought about by heating it with anthron and sulphuric acid. This reaction has been employed for the colorimetric determination of glycerol in aqueous extracts of parchment paper (F. SCHÜTZ, 1938).

Technique of Method.

By means of a pipette 0.2 ml whole blood (or 0.2 ml oxalate plasma) is transferred to 2.5 ml distilled water in a centrifuge tube. Add 0.4 ml basic lead-acetate solution (GOULARD's extract prepared according to PETERS and VAN SLYKE 1932), 0.4 ml of a 5 % solution of zinc sulphate and 0.5 ml of 0.85 n. sodium hydroxyde. Shake after adding each component. Centrifuge after standing at least five minutes. Decant the clear, colourless fluid into a conical centrifuge tube. At this stage the samples may be stored in the refrigerator for 24 hours without affecting the result of the analysis. The excess lead is removed by adding a small drop of saturated carbonate of soda and renewed cen-

trifuging. Of the clear fluid transfer 2 ml by pipette into a Pyrex test-tube, and add 4 ml of a fresh solution of anthron[1] in concentrated sulphuric acid (0.1 mg per ml). Add the same quantity to 2 ml standard solution (0.5—2.0 or 5.0 mg%) and to 2 ml water. After thorough mixing cover the tubes with tinfoil and place for 10 minutes in an oil bath maintained at 170—175°. This will produce a beautiful red-orange colour with green fluorescence when glycerol is present. Compare colorimetrically according to BÜRKER's principle. The precipitation fluids have a low blank value (0.1—0.2 mg%), which is determined separately and subtracted.

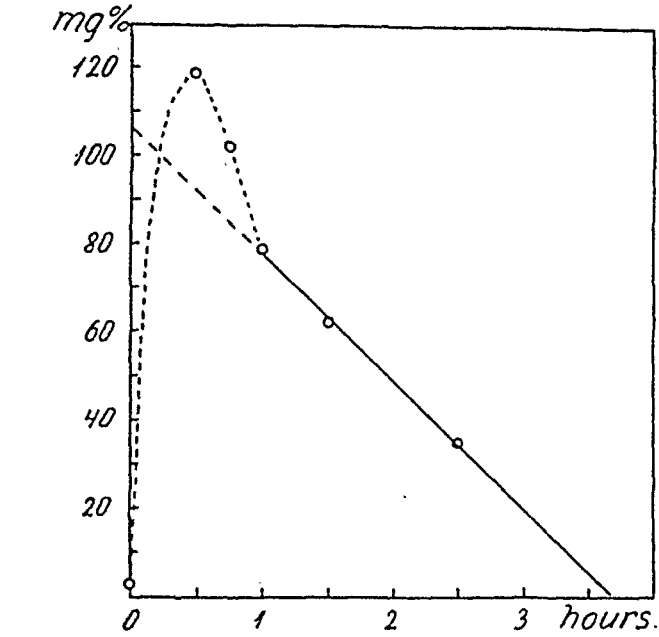
In experiments to find known quantities of glycerol added to blood the mean error of a single analysis is found to lie at about 3 %.

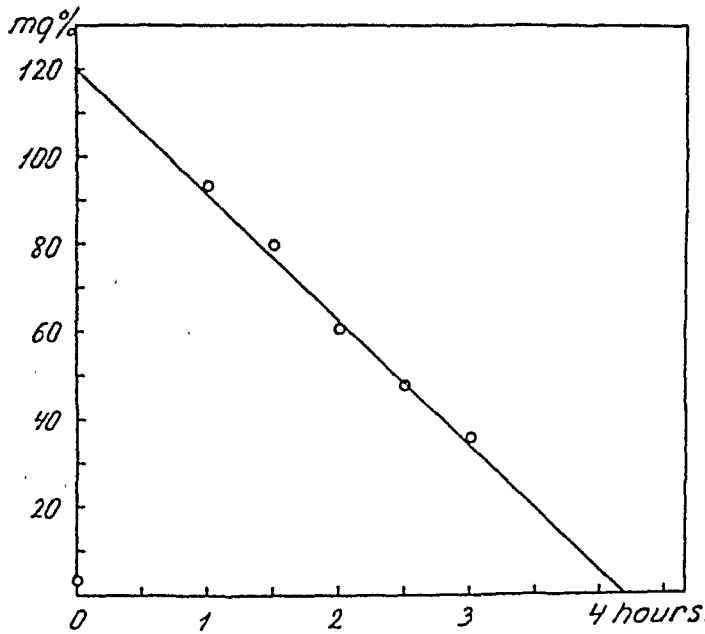
There being good reasons for believing that the liver plays a role in glycerol metabolism, I first experimented with a series of glycerol administrations both to patients with a healthy liver and to liver patients, for the purpose of determing whether the rate of decrease in blood glycerol after glycerol administration if of any value as a liver-function test.

The technique was as follows: To the fasting patient I administered 0.54 g glycerine per kilogramme. (In practice I gave the patients ½ ml glycerinum bidestilatum "Merck" pro kg body weight). It was administered in ten times the volume of water. A blood sample was taken from the ear just before and five times in the course of 2½—3 hours after the glycerol administration.

The blood glycerol curve (figs. 1 and 2) describes a course that is reminiscent of a blood alcohol curve after alcohol administration, as in all cases the concentration in the blood after one hour decreases quite rectilinearly. Consequently it must be justifiable — as with alcohol — to assume that absorption and distribution in the organism are complete after one hour, so that the first part of the curve may be regarded as the resultant of absorption, distribution and elimination, whereas the remaining part is purely an elimination curve. This can be characterized by its rate of descent and its point of intersection with the ordinate. The first expressed as the fall in concentration in per mile per minute corresponds to WIDMARK's factor β (WIDMARK 1932). The second indicates the concentration that would have been obtained immediately after the glycerol ingestion if absorption

[1] Anthron is prepared by means of a weak reduction of anthrachinon in acetic acid solution with granulated tin and fuming nitric acid after LIEBERMANN and GRIMBEL (1887).





Figs. 1 and 2. Glycerol Concentration of the Blood in two Patients after Oral Ingestion of Glycerol.

and distribution had proceeded instantaneously. From this figure it is possible to calculate what fraction of the total mass of the organism acts a a solvent for glycerol like the blood. This frac-

tion, which after WIDMARK is denoted by r, is calculated according to the equation

$$G = p \cdot r \cdot c_0$$

Where G = the quantity of glycerol in g,
p = the body weight in kg,
c_0 = the concentration in ‰ at 0 hours.

Table 1.

Glycerol Ingestion 0.54 g/kg Except Patient No. 6, who received 0.45 g/kg.

No.	Weight kg	C_0 ‰	r	β ‰	r · β g/kg/M	Diagnosis	Age	Sex
1	68.5	1.18	0.46	0.0019	0.0023	Sciatica	41	♀
2	70.8	1.08	0.50	0.0048	0.0024	Sciatica	36	♂
3	63.2	1.10	0.49	0.0052	0.0025	Sciatica	37	♂
4	57.0	1.14	0.48	0.0057	0.0027	Myalgia	48	♀
5	65.1	1.20	0.45	0.0049	0.0022	Mb. Basedowii	29	♀
6	58.0	(0.80)	0.56	0.0051	0.0029	Mb. Basedowii	65	♀
7	60.0	0.94	0.58	0.0056	0.0032	Diabetes mellitus	30	♀
8	74.0	1.21	0.45	0.0054	0.0024	Obstructive jaundice	53	♂
9	54.0	1.04	0.52	0.0048	0.0025	Sub-icterus	44	♀
10	63.4	0.96	0.56	0.0048	0.0027	Acute hepatitis	43	♀
11	58.1	0.94	0.58	0.0045	0.0026	Cirrhosis hepatis	55	♀
12	54.3	0.88	0.62	0.0035	0.0022	Cirrhosis hepatis	58	♂

In addition to C_0, r and β Table 1 contains the velocity of elimination r · β expressed in g/kg/min. Taking all the tests together it will be seen that the mean of r is 0.52 with a dispersion that is at any rate no greater than for alcohol. For alcohol the average for both sexes is 0.61. The average values for β are 0.0049 and 0.0025 respectively, so that the velocity of metabolism in g/kg/min. is 0.0026 for glycerol and 0.0015 for alcohol. No account is taken here of the excretion, which for alcohol is insignificant as the alcohol will be found in the same concentration in the urine as in the plasma, and the loss of alcohol through the expiration air will be of importance only in cases of excessive ventilation. For glycerol the loss is somewhat larger and amounts to about 10 % of the elimination, as found in experiments 1, 4 and 12, where the urine excretion in the period from 60 to 180 minutes was determined at 0.00025, 0.00024 and 0.00026 g/kg/min. respectively.

It is also to be seen from these experiments that there is no indication that the glycerol curve would be clinically useful as a

liver function test (cp. Tests 1—4 with 8—12). Of the only two cases of hyperthyreoidism one has a high, the other a low velocity of metabolism. The highest velocity observed was that of patient No. 7, who has diabetes. This calls for further experiments on diabetics.

These results also call for a further comparison between glycerol and alcohol metabolism. LUNDSGAARD (1937), partly by means of artificially perfused isolated livers and hind-leg preparations, partly with the aid of exviscerated animals, has shown that the alcohol oxydation takes place almost exclusively in the liver and that the oxydation of alcohol does not take place in the muscles. He has also shown that the alcohol metabolism in the liver is a partial oxydation which leads to the formation of an organic acid, probably acetic acid, which presumably is further oxydated in the tissues.

The following experiments are a partial repetition of LUNDSGAARD's experiments with glycerol instead of alcohol. Cats narcotized with chloralose (5 cg/kg) were used in these experiments.

Table 2.

Glycerol Experiments on Cats.

No.	Weight kg	r	β	Elimination	
				g/kg/min.	mg/min.
1	3.4	0.54	0.0059	0.0032	10.7
2	3.8	0.45	0.0081	0.0036	13.9
3	2.7	0.57	0	0	0
4	2.0	0.50	0	0	0
5	2.5	0.52	0.0008	0.0004	1.0

Cats Nos. 1 and 2 were nephrectomized, partly to eliminate any loss of glycerol through the urine, partly that they might serve as a better basis for comparison with Cats Nos. 3, 4 and 5, which were abdominally exviscerated. All the animals received 1.2 g glycerol intravenously at 0 hours except No. 6, which was exviscerated only and served for control purposes.

It will be seen from experiments 1 and 2 that the rate of metabolism was again constant and independent of the blood concentration. In one test (No. 2) it can be seen that even after 10 minutes there was no even distribution in the animal; it can also be seen that at very low concentrations the rate of decrease does not follow the straight line curve but becomes slower. The

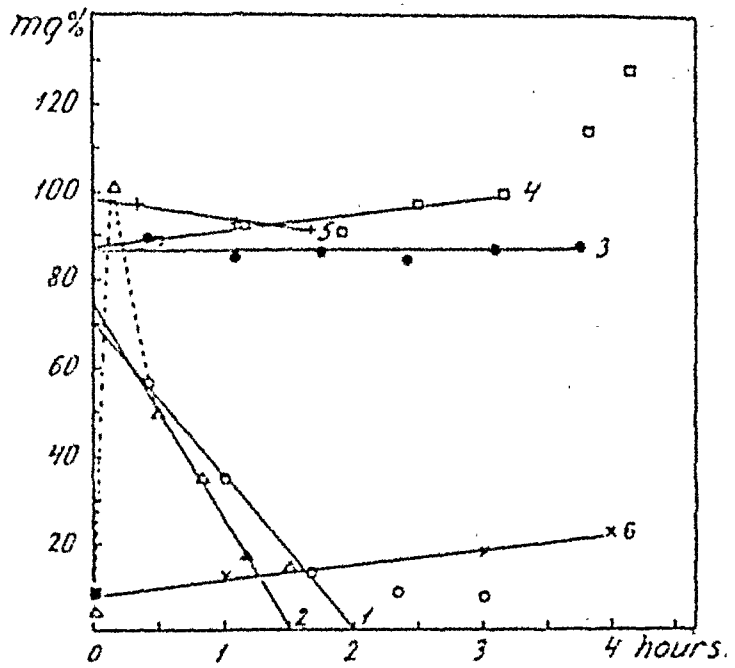


Fig. 3. Elimination Curves from the Experiments in Table 2.

quantity of glycerol remaining, which was thus catabolized more slowly is so small in proportion to the administered quantity that it is of no practical importance to the calculation of the velocity of metabolism, and therefore it is disregarded. The mean r value is the same as for human beings, whereas the rate of metabolism in g/kg/min., i. e. $r \cdot \beta$, is about 30 % higher than for man.

The exviscerated animals do not metabolize glycerol, the blood concentration being unchanged in one case (No. 3), slightly falling in another (No. 5) and slightly rising in a third (No. 4). During the experiment three of the animals (Nos. 3, 4 and 6) received a constant infusion of glucose (10 mg/min.) in order to prevent the fall in blood sugar which otherwise would occur in the hepatectomized animals. These three had increasing blood sugar. However, the increase in the glycerol concentration in the one case cannot be due to the rise of the blood sugar, as I have ascertained by glycerol analyses on blood with various quantities of glucose added, but must be due to an accumulation in the blood of other products as a consequence of the abnormal metabolism. The control experiment (6) also supports this contention.

Accordingly, these experiments show that the glycerol metabolism must, in any case primarily, be dependent on the function of the excised organs, and thus it is natural to suppose the liver as the seat of that metabolism. As I am not master of the technique of liver perfusion experiments, Professor LUNDSGAARD made them for me, and I would here express my thanks to him. The technique was described by LUNDSGAARD, NIELSEN and ØRSKOV (1936). Moreover, the oxygen consumption was determined by a photoelectric measurement of the oxygen in the venous blood, as recently described by LUNDSGAARD (1942). By this means a considerable drop in the oxygen consumption of the liver is observed in the first 30—60 minutes after perfusion begins, whereafter it remains almost constant.

Table 3.

Liver Perfusion Experiment.

No.	1	2	3	4
Rate of glycerol metabolism mg/min. .	3.00	3.49	6.25	7.90
Computed oxygen consumption resuming complete oxydation of glycerol metabolized ml/min.	2.56	2.97	5.33	6.73
Oxygen consumption found ml/min. .	2.00	2.25	3.25	3.35
Liver weight, g	70	78	100	86

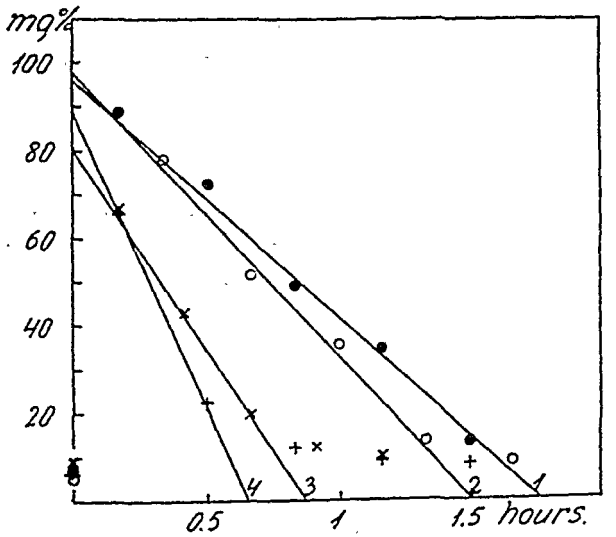


Fig. 4. Elimination Curves for the Experiments in Table 3.

In each test 300 mg glycerol were added to the perfusion blood. In experiments 1 and 2 the addition was not made until after the oxygen consumption had become constant. Here again the curves have a rectilinear course, and the rate of metabolism averages 3.25 mg/min., which is about 25 % of the metabolism in an intact animal. Under corresponding experimental conditions LUNDSGAARD found that the alcohol metabolism in the isolated liver was about 60 % of that of an intact animal and he ascribed this to the initial fall in the oxygen consumption. I therefore added the glycerol shortly after perfusion began, i. e. under decreasing oxygen consumption, the result being that these experiments (Nos. 3 and 4) gave a much higher rate of metabolism for glycerol, about 60 % of that of an intact animal.

The addition of glycerol causes no great increase in the oxygen consumption of the liver. If the catabolized quantity of glycerol were completely oxydized, the oxygen consumed by this alone would clearly exceed the liver's real oxygen consumption, as will appear from Table 3. The oxydation in the liver must, therefore, be incomplete.

A partial oxydation would cause a fall in the respiratory quotient. The most likely partial oxydation would be a conversion to glucose, which requires a much lower consumption of oxygen than for total oxydation (14 %). Provided that the whole of the glycerol is converted into carbo-hydrate without an increase in the total oxygen consumption, everything else being equal, the RQ will be reduced, e. g. from 0.70 to 0.59. In a single test, in which the RQ was determined before and after the administration of the glycerol, I was unable to demonstrate any change in RQ, whereas in LUNDSGAARD's alcohol experiments it fell from 0.69 to 0.37. It should be observed, however, that the fall in the RQ which under the same circumstances one would expect to find when alcohol is oxydated to acetic acid is about four times as great as when glycerol is converted into carbo-hydrate. When it is considered that part of the glycerol is undoubtedly oxydized completely with a respiratory quotient (0.86) which lies above the normal of the isolated liver, it is no wonder that the addition of glycerol results in no distinct change in the quotient. No production of acid can be demonstrated during the glycerol metabolism, the alkali reserve being constant.

Owing to the disagreement between the rate of metabolism in the isolated liver and in the intact animal, the possibility cannot

be excluded that glycerol is also broken down in the intestine. For the purpose of approaching this problem a little nearer an experiment was made by perfusing an isolated cat intestine with blood to which glycerol was added. No definite change was observed in the glycerol concentration, but the intestinal function undoubtedly suffers under perfusion, as oedema sets in rapidly with sanguineous exudation into the lumen. I therefore tried leaving out the liver without compromising the circulation of the intestine. With a G-shaped glass cannula I connected the portal vein and the right renal vein, thus making a kind of Eck's fistula. At the same time the hepatic artery and the right renal artery were ligatured and the left kidney was removed. The animal received heparin intravenously just prior to the setting up of the anastomosis, and glucose was infused constantly as in the case of the exciscerated animals. After the experiment there was no stasis in the portal region, and the intestine had a perfectly natural appearance with no exudation into the lumen.

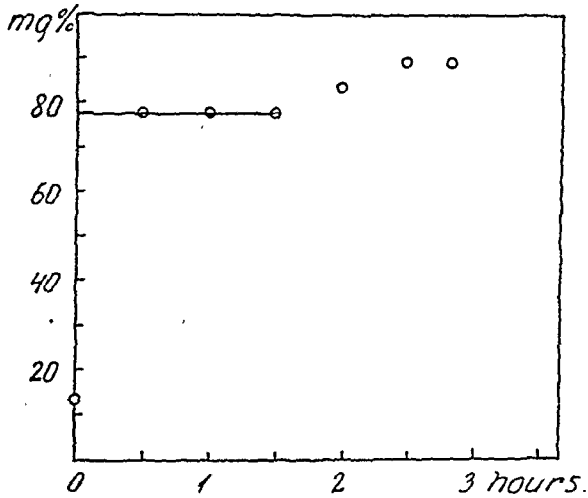


Fig. 5. Elimination Curve of Hepatectomized Cat.

Fig. 5 shows the result of an experiment of this kind. Here again the curve is horizontal at first, whereupon the values rise as in one of the experiments with exviscerated animals. It would seem that in hepatectomized animals a substance accumulates in the blood which has a colour reaction like that of glycerol.

For comparison brief mention may be made of earlier experiments by LEDERER (1936). The glycerol method he used is based

on a reduction of bichromate in sulphuric acid. The so-called chrome index of the plasma is determined by means of titration of the excess bichromate; by subtracting from this the value corresponding to the glucose content the so-called residue index is reached. The increase of the glycerol content is then calculated from the difference between the "residue index" before and after the administration of the glycerol.

Employing this somewhat uncertain method LEDERER studied three intravenous injections of glycerol in dogs, 1 g glycerol per kg; four blood samples were taken during the first 90 minutes. By plotting the results into a coordinate system it is found that two of the curves are rectilinear and have the same inclination, the third one being more irregular. I have calculated the r values at 0.65, 0.44 and 0.33 and the velocity of metabolism at 11.1, 13.7 and 9.4 mg/kg/min., that is to say they are three or four times as high as in my own experiments on man. Perfusion of dog livers by LEDERER gave metabolic values of 7.4 and 29.3 mg/min., but in a control test without glycerol there was a marked rise in the "residue chrome index", so that in reality the metabolism must be assumed to have been much greater. Thus the results of LEDERERS experiments, through carried out with a primitive technique for glycerol determinations correspond in some respects to mine.

Summary.

There is a distinct similarity between the metabolism of glycerol and that of ethylalcohol. This similarity is recognizable by the fact that:

1) The elimination curve is rectilinear, i. e. the rate of glycerol metabolism is constant, and independent of the blood concentration.

2) The glycerol metabolism is localized exclusively, or almost exclusively in the liver.

3) At most a fraction of the glycerol can be completely oxydized in the liver, while at least the larger fraction must be incompletely oxydized.

References.

HOLST, E. J., Glycerinundersøgelser til Belysning af Teorien om Resorptionsfosforyleringen, Diss., Kbhvn, 1943.

LEDERER, J. A., Rev. belge sci. med. 1936, *8*, 373.

LIEBERMANN, C. and A. GIMBEL, Ber. dtsch. chem. Ges. 1887, *20*, 1854.

LUNDSGAARD, E., C. R. Lab. Carlsberg, Ser. chim. 1937, *22*, 333.

LUNDSGAARD, E., Acta physiol. scand. 1942, *4*, 330.

LUNDSGAARD, E., N. A. NIELSEN and S. L. ØRSKOV, Skand. Arch. Physiol. 1936, *73*, 296.

PETERS, J. P. and D. D. VAN SLYKE, Quantitative Clinical Chemistry, London 1932. *2*, 636.

SCHÜTZ, F., Der Papier-Fabrikant 1938, 36, Part I, 55.

WIDMARK, E. M. P., Die theoretischen Grundlagen und die praktische Verwendbarkeit der gerichtlich-medizinischen Alkoholbestimmung. Berlin 1932.

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The Influence on Growth and Metabolism of the Relation between the Proportion of Proteins and of Aneurin and Lactoflavin in Food.

By

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Introduction.

In 1937 HAMMARSTEN published a series of animal experiments, for which the control animals had been reared on a well-defined diet, nr 211, producing fine and well-grown animals. This diet contained 15 % casein as the primary source of proteins. In order to obtain optimal growth a further 5 % casein was given to the pregnant and lactating females and to the young animals at the time when growth was most rapid. BORGSTRÖM (1941) used a modification of diet 211 and obtained still better and stronger animals. When comparing BORGSTRÖM's diet and nr 211 we find that the latter must have contained a "sub-optimal" amount of one or more of the B vitamins (aneurin or lactoflavin). Without increasing the protein content (15 % casein) BORGSTRÖM by augmenting the supply of vitamins B_1 and B_2 thus produced better animals than HAMMARSTEN. A comparison between the growth results from these two diets shows that there is a possibility of a plentiful supply of B vitamins facilitating the protein metabolism to such a degree that a smaller protein content is sufficient to obtain optimal growth. This problem, important from the nutri-

tive and physiological as well as from the economical point of view, will be dealt with below.

The aim of the present study has thus been to investigate the possibility of obtaining optimal growth by means of an ample supply of B vitamins with a relatively low protein content. In our country a suitable diet should, from an economical point of view, furnish the greater part of the energy supply in form of carbohydrates. We know by experience that a diet, rich in carbohydrates, requires above all a good supply of B_1 vitamin. As aneurin in its function is bound up with lactoflavin as well as with nicotinic acid amide, it is necessary to provide a sufficient supply also of those vitamins.

Experimental Data.

Growth Experiments. Series A.

Mixed diets were prepared for 5 groups of animals. The ingredients were bought at the same time. The basic diet A of group I corresponds to diet 1 b in BORGSTRÖM's above-mentioned study (See Table I).

Diet II of group II: Basic diet A + 2 mg aneurin-hydro-chloride/kg food.
» III » » III: » » A + 2.5 mg lactoflavin/kg »
» IV » » IV: » » A + Vit. B_1 + Vit. B_2 as in II and III resp.
» V » » V » » A + addition of 5 % casein.

Table I.

Composition of diets in series A.

	Basic diet A = Diet I Parts by weight	Diet V Parts by weight
Rice meal	696	646
Casein	150	200
Peanut oil	37	37
Cod-liver oil "Leo"	3	3
Wheat germ	30	30
Sugar	18.6	18.6
Hip meal	5	5
Brewer's yeast (dry)	30	30
Salt mixture	30.4	30.4
	1 000	1 000

Table II.

Salt mixture.

	Parts by weight
Sodium chloride (NaCl)	83
Sodium sulphate (Na_2SO_4, 10 H_2O)	148
Prim. potassium phosphate (KH_2PO_4)	78.1
Calcium lactate	250
Ferric citrate	35
Magnesium oxide (MgO)	48
	642

The percentage of nitrogen, calcium and magnesium of the diets in mg/gr dried food was:

	Diets I—IV	Diet V
N	37.82	44.84
Ca	2.52	2.81
Mg	1.19	1.43

(Elementary analyses performed after wet combustion.)

For estimation of the vitamin content in the basic diet see account by HAMMARSTEN (1937).

Strong female rats (albinos) of the stock from the Institute of Medical Chemistry were chosen and covered simultaneously. Among their litters 20 young females of the same size were chosen for each group. Individuals from each separate litter were distributed among the various groups. From the age of 4 weeks they were fed on the various food mixtures. The food was mixed with water to form a dough, and the animals were allowed to eat as much as they wanted. They were given tap-water to drink. The rates of growth were observed. During a period of 20 weeks every animal was put into the "metabolism cage" for three weeks. During this time the amount of food supplied was weighed and the faeces and the urine were collected for analysis during the last two weeks. One pair of animals from each group was thus observed for three weeks until all the animals had been observed. At the end of the investigation we found, however, that all the groups had grown equally. The experiment had started in the summer of 1941. At this time there were already great difficulties in finding sufficiently pure ingredients for the diets. The only way of explaining the results of the experiment is to assume that the basic diet A already contained an optimal — or nearly optimal — amount of B vitamins. They further indicate that an augmented supply of proteins has not produced any better growth. In order to check that the increase in weight was not due to an increased accumulation of fat in any one group the increase in length was also measured. The quotient — weight: length of back — gave corresponding values for all groups (length of back being the distance from nose to root of tail). The age of the animals at the end of the experiment was 36 weeks.

Table III.

Series A.

Group	Weight in grams M	εM	n	Quotient weight to length of back
I	239	± 9.0	20	10.7 ± 0.31
II	245	± 8.4	20	10.8 ± 0.31
III	240	± 5.6	20	10.5 ± 0.19
IV	240	± 4.4	20	10.5 ± 0.19
V	240	± 5.9	19	10.6 ± 0.20

We have not made a closer analysis as to the Ca and Mg balance of the results from the metabolism experiment in series A, as a rough estimation showed that we got similar results there too. The nitrogen metabolism from the balance experiments is seen in Table IV.

Although the final weights of the animals were the same and the amount of food consumed had been the same during the balance experiments some differences in the nitrogen balance between groups III and V can be detected. In group III, which had been given an extra supply of lactoflavin, the retention of nitrogen was greatest. Group V, the food of which contained 5 % more casein than the other groups', has retained, absolutely taken, exactly the same amount of nitrogen as group I. This amount of nitrogen is thus either optimal for the metabolism of the animals or represents the maximum amount of nitrogen which can be digested with regard to the composition of the food (f. ex. the amount of vitamin B). When calculated in per cent the nitrogen retention in group V is however lower than in group I. That these animals burn more N products is evident from the higher nitrogen values in the urine. As regards group V one might thus talk about a superfluous consumption of proteins. It is possible that the growth of group V might be still further increased over that of group I by giving an additional supply of vitamins B_1 and B_2 together with the higher quantity of proteins. This question can only be decided by further experiments. It should be kept in mind, however, that hereditary factors finally exert a limiting influence on optimal growth even under the best growth-inducing external conditions.

The faecal output of nitrogen in per cent of the nitrogen intake is for all the groups of about the same quantity. The differences become still less if the N values are expressed in per cent of faeces

Table IV.

Series A. Nitrogen metabolism during balance experiments of 20 weeks, beginning at the age of 15 weeks, 20 animals in each of the groups I—IV, 19 animals in group V.

Group	Food consumed in grams	Amount of N supply in grams	Nitrogen in % of amount supplied				Nitrogen in % of amount of fæces	»Retention» absolute amounts of nitrogen in grams
			Urine M ± M	n	Fæces	»Retention»		
I	4394	166.185	70.0 ± 1.5	39	6.9	23.1	6.75	39.88
II	4404	166.540	68.3 ± 1.6	39	6.9	24.8	6.64	41.47
III	4348	164 423	66.0 ± 1.4	38	6.8	27.2	6.62	47.02
IV	4442	168.136	68.5 ± 1.2	38	6.7	24.8	6.50	43.55
V	4164 / [1] 208	175.757	72.5 ± 1.2	38	5.8	21.7	6.21	38.15 / [1] 1.91

[1] 5 % of the above value which ought to be added on comparison with the values of the other groups in columns 2 and 8. Cf number of animals above!

Differences in uric nitrogen values (column 4):

I—V	I—III	III—V	IV—V
− 2.5 ± 1.9	4.0 ± 2.05	− 6.5 ± 1.84	− 4.0 ± 1.7

excreted. Faecal nitrogen is composed of three main components: Non-absorbed nitrogen from the food, nitrogen from the digestive secretions and nitrogen originating from the intestinal bacteria. As it is impossible to decide the inter-relations of these components within the different groups from the present data, we have refrained from a statistical arrangement of them. The values given here show, however, that there cannot have been any real difference between the various groups regarding the intestinal enzymatic procedures or the absorption. We have on the other hand worked out the values of nitrogen in urine, expressed in per cent of the nitrogen consumption. These values show how much nitrogen has been burned by the organism, and are thus of greater importance than the faecal nitrogen values. We are fully aware that the metabolism also includes the nitrogeneous substances of the body proper and not only those given with the food. If the metabolism of nitrogeneous substances is calculated in the above-mentioned way, we find a statistically significant difference between groups I and III (Diff. $= 4.0 \pm 2.05$), groups III and V (Diff. $= -6.5 \pm 1.84$) and groups IV and V (Diff. $= -4.0 \pm \pm 1.7$). The nitrogeneous ingredients of the food are best utilized and best retained in group III (additional supply of lactoflavin) just as the relatively highest metabolism and lowest retention is found in group V.

These experiments show the conditions for nitrogen metabolism in the grown animal. The main period of growth was over before the beginning of the balance experiments.

Growth Experiments. Series B.

In autumn 1942 a new series of animals — Series B — including 50 young female rats 4 weeks old, was put up and divided into 5 groups. By excluding wheat germ and yeast the basic diet was made to contain a considerably smaller amount of B vitamins. For the rest this diet — basic diet B — is composed of the same ingredients as basic diet A.

The experiment was conducted as in Series A, that is the young rats were taken from their mothers when 4 weeks old and divided into different groups. The growth experiments proceeded for 20 weeks, at the end of which the animals were about 170 days old. This time the experiments showed a clear difference in growth as is seen from Table VI.

Table V a.

Composition of diets in series B.

	Basic diet B = diet VI Parts by weight	Diet X Parts by weight
Rice meal	696	646
Casein	150	200
Peanut oil	37	37
Cod-liver oil "Leo"	3	3
Sugar	18.6	18.6
Hip meal	5	5
Salt mixture (See Table II)	30.4	30.4
	940	940

Table V b.

Composition of diets in series B.

Diet nr

VI Basic diet B
VII » » B + 4.0 mg aneurin-hydro-chloride/kg food
VIII » » B + 5.0 mg lactoflavin/kg
IX » » B + B_1 as in VII and B_2 as in VIII
X » » B + additional supply of 5 % casein.

Table VI.

Series B. In each group 10 animals.

Weight of animals at different ages, grams. Food consumed and growth per gram of food consumed.

Group:	VI	VII	VIII	IX	X
Diet:	B	B + aneurin	B + lacto-flavin	B + aneurin + lacto-flavin	B + 5% casein
Age in weeks		Weight, M ± sM.			
4	49 ± 2.1	49 ± 2.2	49 ± 1.9	49 ± 2.1	49 ± 2.4
8	97 ± 4.4	99 ± 3.6	120 ± 4.1	119 ± 6.2	85 ± 5.2
12	136 ± 6.2	138 ± 4.2	175 ± 5.4	166 ± 6.8	116 ± 4.6
16	158 ± 5.9	160 ± 5.3	199 ± 5.7	183 ± 7.8	142 ± 6.1
20	170 ± 6.9	176 ± 7.7	218 ± 7.8	194 ± 8.6	153 ± 6.8
24	174 ± 5.9	183 ± 7.7	226 ± 8.2	200 ± 8.2	162 ± 4.5
Increase in weight, g.	125 ± 4.7	134 ± 7.5	177 ± 7.9	151 ± 7.1	113 ± 5.2
Total amount of food consumed, kg.	16.50	20.00	20.38	18.70	15.06
Growth in mg per gram of food cons.	75.6 ± 2.84	66.7 ± 3.76	86.8 ± 3.91	80.4 ± 3.80	74.9 ± 3.45

If the growth curves and the weight of the animals at different times are compared, it is evident that lactoflavin is the "limiting factor" of diet B. An augmented supply of aneurin (group VII) does not increase the weight of the animals until the end of the experimental period, when the animals in group VII have attained a slightly higher weight (Diff. 9 ± 9.7), but this difference is not statistically significant. The difference in weight is only apparent towards the end of the investigation and may thus be due to a varying accumulation of fat. If the "limiting factor" of group VI, lactoflavin, is added, we obtain differences (group VI—group VIII) that are statistically significant from the age of 10 weeks. If a relative surplus of aneurin is added and the lactoflavin supply is kept high (group IX) growth will again decrease. (Difference at the age of 24 weeks [groups VIII—IX] is 26 ± 11.6, i. e. shows a statistically good significance.) *Best growth is thus obtained by an adequate and well balanced supply of aneurin and lactoflavin.*

If the casein content of the food is increased from 15 to 20 % (i. e. about 25 % proteins in all) without augmenting the supply of B vitamin, *growth decreases.* At the end of the investigation period the difference in growth between groups VI and X is 12 ± 7.4, which although not really significant statistically is nevertheless so great that it cannot be disregarded. When comparing the growth of these last-mentioned groups at the age of 12, 16 and 20 weeks respectively we find statistically more satisfactory differences (20 ± 7.7, 16 ± 8.5 and 17 ± 9.7 resp.).

The corresponding figures for growth in Series A, reared on a larger supply of B vitamin, have unfortunately been lost for the period between 4 and 13 weeks. The weight curves of group I (diet containing 15 % casein and about 20 % total protein) and group V (diet containing 20 % casein and about 25 % total protein) for the period 14 to 24 weeks coincide exactly. While an increase of total protein in the food from 20 to 25 % together with a small quantity of lactoflavin lowers the power of growth, an increased supply of B_2 vitamin will give exactly the same growth in both cases. (Basic diet B lacks wheat germ and yeast, which are included in basic diet A; otherwise both diets are similar. Basic diet B gives after addition of lactoflavin and within the margin of error just as good or even slightly better growth than basic diet A. The amount of other necessary growth factors in basic diet B thus seems to be sufficient.) On a high B vitamin standard (Series A), the animals seem able to balance the additional

supply of protein without any danger, but they cannot utilize it to increase growth. *Our experiments thus show that an optimal and well balanced supply of aneurin and lactoflavin is more important than a high protein standard and further that a high protein standard requires a high B vitamin standard.* These observations are confirmed from other sources: the capacity of strict vegetarians to manage without animal protein and the good fodder properties of the AIV silage might be ascribed to a high standard of B vitamin supply.

The amounts of food consumed during the whole time of the experiment and the growth per gram of food consumed will be found in Table VI.

When we examine growth per gram of food consumed we find the differences which are given in Table VII.

Table VII.

Groups	Growth per gram of food consumed	
	Difference $\frac{\Delta}{M \pm \varepsilon M}$ mg	Significance $\frac{\Delta}{\varepsilon M}$
VI —VII	8.9 ± 4.71	1.89
VI —VIII	− 11.2 ± 4.83	*2.32*
VI —IX	− 4.8 ± 4.74	1.00
VI —X	0.7 ± 4.47	0.16
VII —VIII	− 20.1 ± 5.42	*3.70*
VII —IX	− 13.7 ± 5.35	*2.56*
VII —X	− 8.2 ± 5.10	1.61
VIII—IX	6.4 ± 5.45	1.17
VIII—X	11.9 ± 5.21	*2.28*
IX —X	5.5 ± 5.13	1.07

Significance good: $\Delta = 2 \times \varepsilon\Delta$
» very good: $\Delta = 2.5 \times \varepsilon\Delta$
» certain: $\Delta \geqq 3 \times \varepsilon\Delta$

As was to be expected an increased supply of aneurin stimulates considerably the appetite of the animals. But growth per gram of food supplied decreases (significance only 1.89, though). Supply of lactoflavin only (group VIII) stimulates appetite just as much as the supply of aneurin in group VII and gives increased growth per gram of food consumed. When supplying both B_1 and B_2

vitamins (group IX, where the B_1 supply dominates but B_2 is supplied as in group VIII) the animals consume less food than in groups VII and VIII but more than group VI. Growth per gram of food consumed is somewhat greater than in group VI but the difference is not statistically certain (probability only 68 %). In group VIII we find the highest absolute growth and also the best growth per gram of food consumed. The difference between group VIII on the one hand and groups VI, VII and X on the other shows a very good or statistically certain significance. Between groups VIII and IX no certain difference is to be found. An increase in B_1 supply does not improve the growth to a further degree than that found in group VIII.

As has been mentioned earlier lactoflavin was the limiting factor in diet VI. This vitamin is thus as important for growth as aneurin. From the economical point of view the experiment demonstrates the importance of finding the possible limiting factor in the food of a given food budget. It is true that an increase of the already sufficient aneurin content stimulated the appetite of the animals (See group VII). But growth per gram of food consumed is less than in the control group VI. An adjustment of the B vitamin content of the food by means of increased supply of lactoflavin does on the other hand give both the best absolute growth and the best growth per food unit supplied. That is, a sufficient and well balanced supply of aneurin and lactoflavin will result in not only the best growth but also the best economical utilization of the food. Or in other words: By adding its limiting factor to the food we attain the maximal utilization of the food-stuffs supplied.

Metabolism Experiments. Series B.

Nitrogen, Calcium and Magnesium Metabolism.

At the age of 6 weeks one pair from each group was put into the "metabolism cage". These metabolic experiments lasted for three weeks, of which the two later weeks were the real period of experiment; during the first week the animals had to adapt themselves to the experimental conditions. Two weeks after the first pair another was put into a "metabolism cage" and so on until all the animals had been examined. The amount of food consumed was noted and, during the two latter weeks, urine and faeces

Table

Retention of calcium, magnesium and nitrogen a) in per

Group		VI				VII			
Age in weeks at the beginning of the experiment		Weight	Ca	Mg	N	Weight	Ca	Mg	N
7	a	91	84.5	35.5	48.6	89	83	46	46.2
	b		1.50	0.57	11.2		1.44	0.72	10.2
8	a	104	95	36	33.3	102	84.5	34	25.7
	b		1.49	0.49	6.6		1.25	0.45	4.9
9	a	107	69	37	45.7	94	79	34.5	(40.0)
	b		1,00	0.50	8.6		1.16	0.45	(9.3)
10	a	117	64.5	(6.5)	49.4	103	61	(5.0)	(56.7)
	b		0.86	(0.08)	8.7		0.85	(0.06)	(10.7)
11	a	109	55	(− 51)	32.6	123	47.5	(− 43.5)	32.6
	b		0.88	(− 0.67)	6.7		0.67	(− 0.52)	6.2
12	a	115	58	(17.5)	41.2	129	52	11.5	31.8
	b		0.88	0.23	8.0		0.64	0.13	5.4
13	a	150	53	20	34.0	136	40	− 2	32.8
	b		0.72	0.28	5.9		0.52	− 0.02	5.4
14	a	132	44.5	24.5	31.2	141	46	8.5	34.1
	b		0.57	0.27	5.1		0.55	0.10	5.2
15	a	136	36.5	28.5	30.9	149	25.5	26	21.2
	b		0.47	0.32	5.0		0.29	0.26	3.1
16	a	149	26.5	19	21.0	168	28	28	37.0
	b		0.33	0.20	3.3		0.35	0.29	6.1
17	a	154	31.5	12.5	7.5	169	26.5	22	11.5
	b		0.46	0.15	1.4		0.36	0.23	2.1

[1] Bracket denotes analysis values of the same pair of animals within the respective

VIII.

cent of amount supplied, b) in mg, gram body weight.

VIII				IX				X			
Weight	Ca	Mg	N	Weight	Ca	Mg	N	Weight	Ca	Mg	N
102	81	43.5	35.7	103	81.5	29.5	39.1	88	61	35.5	35.3
	1.46	0.72	8.2		1.39	0.48	8.6		1.71	0.57	8.8
120	78.5	31	30.8	126	83.5	25	31.5	96	60.5	21	20.9
	1.22	0.45	6.1		1.15	0.32	5.6		0.99	0.26	4.4
125	76	13	36.2	132	81	30	45.3	101	58.5	17.5	20.4
	1.10	0.17	6.8		1.20	0.41	8.7		1.00	0.24	4.4
142	67.5	(— 11.0)	37.9	150	66.5	(— 5)	39.0	108	50	(2)	38.8
	0.94	(— 0.14)	3.5		0.95	(— 0.06)	7.2		0.81	(0.02)	7.8
143	50.5	(— 22)	29.5	131	54	(— 38)	31.8	97	36.5	(— 23.5)	37.2
	0.70	(— 0.22)	5.2		0.77	(— 0.46)	6.0		0.56	(— 0.17)	2.0
155	(34)	19	33.8	144	47	3.5	28.9	100	(28.5)	25	22.9
	(0.43)	0.21	5.5		0.26	0.03	4.9		(0.40)	0.27	2.0
160	43	7	26.9	164	32	4	28.4	107	48.5	43.5	(41.5)
	0.53	0.07	4.3		0.40	0.04	4.5		0.87	0.64	(9.5)
169	40	22.5	33.2	168	34.5	25.5	20.0	114	43	49.5	28.5
	0.47	0.23	5.1		0.43	0.25	3.2		0.74	0.70	6.2
179	20	29.5	35.4	176	20	20	28.3	125	42.5	37.5	39.7
	0.21	0.29	5.2		0.22	0.19	3.9		0.63	0.48	6.3
203	25.5	23	7.1	157	40	12	30.8	147	45	34	30.4
	0.30	0.22	1.0		0.48	0.13	4.7		0.64	0.38	3.0
213	16.5	22.5	(50.7)	170	26.5	17.5	35.8	154	33	19.5	(42.7)
	0.31	0.21	(7.5)		0.33	0.17	5.7		0.49	0.21	8.0

groups.

were collected. The nitrogen metabolism was the head object of our investigation but the calcium and magnesium metabolism was also checked, as we wanted to detect a possible influence of the differences in B vitamin and nitrogen content of the various diets on the mineral metabolism. The values are to be found in Table VIII.

The age of the animal at the beginning of the experiment week is given in weeks. The weight is the medium of the weight of each pair of animals at the beginning of the respective weeks of experiment. "Retention" denotes the amounts of nitrogen, calcium and magnesium not recovered in urine and faeces and expressed in per cent of the N, Ca and Mg amounts supplied. Losses of nitrogen from hair or epithelium have not been taken into account. The urine has been protected from putrefaction by adding toluene. In some rare cases we have had reason to suspect bacterial decomposition of the urine accompanied by loss of nitrogen and too high retention values. These values are marked ( ) in the tables and excluded from the calculations.

The divergences between the retention values from a pair of animals are often greater than the divergences between the values from different groups. There is no certain difference to be found between the values obtained from different groups with the possible exception of the nitrogen and the calcium and magnesium metabolism within group X. In this group the calcium and magnesium retention, when compared to that of group VI, is lower during the first half of the experiment and higher during the last three weeks. The higher protein content of diet X gives an increased amount of acid metabolic residues which when secreted necessitate an increased amount of bases in order to neutralize. Considering the good base-conserving function of the rat kidney it is to be expected that this neutralizing will have taken place mainly by means of ammonia. (Unfortunately the NH_3 secretion has not been investigated.) About 3.6 % of the calcium supplied is recovered in the urine in group VI — the corresponding secretion in group X amounts to 4.0 %. The difference is not statistically significant. As has been already mentioned, the difference in retention is greater during the former half of the experiment. During the first 6 weeks the animals in group X secrete in the urine on an average 3.45 % calcium and 22.6 % magnesium while the corresponding values in group VI are 2.7 % calcium and 30 % magnesium. The greatest difference is found between

the faecal contents of calcium and magnesium. Calculating on the amounts supplied we find the following values:

Faeces from group VI contain 26.4 % Ca and 56.4 % Mg
» » » X » 46.1 % Ca » 64.5 % Mg.

There can scarcely have been any reason for a decreased resorption, which is further contradicted by the fact that group X absorbs more calcium and magnesium during the last weeks of the experiment than does group VI.

NICOLAYSEN has shown the improbability of any excretion of calcium through the intestinal wall. According to him the calcium found in faeces consists partly of unabsorbed calcium and partly of calcium secreted with the digestive juices. A probable explanation of the low calcium and magnesium retention in group X during the former half of the experiment we think is found in the following theory: The higher amount of proteins in the food causes an increased secretion of digestive juices. The lively metabolism in the growing animal means that it converts more food per unit body weight and thus secretes relatively more digestive juices than the grown animal. Now if the animal has been unable to fill its calcium depots during the time when growth is most rapid, as for example in group X, it will try to do so later, when the metabolism per unit body weight is not so lively any longer. Further experiments with a greater number of animals, the balances of which have been followed during the whole time of growth, are necessary in order to settle this question definitely.

If the retention is calculated in mg per gram of growth we find that groups VII, VIII and IX, i. e. the animals with the best growth, retain an equal amount per period of both nitrogen and of calcium and magnesium. When these three groups are compared with group VI, no difference is to be found as to the retention of nitrogen and magnesium. The calcium retention is somewhat higher in group VI, especially during the earlier half of the experiment. In group X we find as has been stated earlier that the calcium and magnesium retention is lower during the former half of the experiment and, reversely, higher during its latter half, when compared to the values from group VI.

If the nitrogen retention and the nitrogen content in urine and faeces in the different groups are compared at the time of the balance experiment, we find the following values: (See table IX.)

Table IX.

Nitrogen metabolism during balance experiments.

Group	(Age of animals = 7—17 weeks)			
	N supply in grams	Per cent of amount supplied		»Retention»
		Urine N M ± εM n	Faecal N	
VI	51.613	60.6 ± 3.16 22	5.1	34.3
VII	51.741	61.8 ± 3.70 22	5.4	32.8
VIII	61.865	62.2 ± 2.70 22	5.0	32.8
IX	59.882	62,5 ± 2.20 22	5.3	32.2
X	52.544	67.8 ± 3.16 22	5.2	27.0

Diff. (X—VI) = 7.2 ± 4.47.

A dispersion curve with oblique distribution might here have been expected, but this is not the case. The dispersion is as a matter of fact so great that it more than covers the possible greater absorption which one expects during the beginning of the experiment.

All the five groups have proportionally the same low amount of faecal nitrogen, which indicates that the enzymatic processes in the intestines and the absorption have taken place with equal completeness in all groups. The differences found must thus be due to the metabolism within the respective groups. As the animals have been allowed to eat *ad libitum*, growth must have been controlled by the appetite in groups VI—IX. Group X with a 5 % higher supply of pro'ein in its food has burned a part of the protein consumed (or more of it than the other groups). (The significance of the difference between groups VI and X is only 1.61, though.) That is, group X secretes more nitrogen with the urine and retains less nitrogen than the other groups.

A means of measuring the calcium retention of the animals is to examine the calcium content of the skeleton. At the end of the experiment the animals were killed, one pair at a time in the same order as in the metabolism experiments. Thus one pair from each group was killed the same day. The femora were cut out and their calcium content determined. The results are given in Table X.

Table X.

Group	Calcium content of the femur in % of clean-scraped, dried bone		
	M	εM	n
VI	22.4	± 0.22	20
VII	22.4	± 0.34	19
VIII	22.7	± 0.26	20
IX	22.3	± 0.26	19
X	22.0	± 0.25	19

All the groups thus show the same content of calcium in their bones.

The investigation will be continued with experiments on the protein minimum at various B vitamin standards.

The nitrogen analyses have been made according to NORMAN-JENSEN's KJELDAHL method, the calcium analyses according to HAMMARSTEN's modification of ARON's method and the magnesium analyses according to STOLBA. As to the accuracy of the methods we refer to earlier works by HAMMARSTEN (1937) and BORGSTRÖM (1941). 2 072 elementary analyses have been performed. For want of space the primary values cannot be given here.

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Summary.

Growth experiments have been performed on two series (A and B) of 100 and 50 albino rats (females). In each series the animals have been distributed on 5 groups with a varied supply of aneurin and lactoflavin in 4 groups and an extra supply of 5 % casein in the 5th group. Other B vitamins have been given in sufficient amounts. The diets have contained a high percentage of A and D vitamins

The following results have been obtained: Lactoflavin is as important a growth factor as aneurin and stimulates the food consumption of the animals to the same degree. Both vitamins are necessary not only for the carbohydrate metabolism but also for the protein metabolism. If the protein supply is increased from 20 to 25 % at a relatively low standard of lactoflavin supply, growth decreases. A high protein standard necessitates a high B vitamin supply. An optimal and mutually balanced supply of aneurin and lactoflavin is more important for growth than a high protein standard. A sufficient and well balanced supply of aneurin and lactoflavin together with a sufficient but not too great supply of proteins gives the best growth and the best economical utilization of the food.

Literature.

BORGSTRÖM, S., Acta Physiol. Scand. 1941. *2.* Suppl. VII.
HAMMARSTEN, G., Lunds Univ. Årsskrift. N. F. 1937 Avd. 2. 32. Nr 12.
—, Skand. Arch. Physiol. 1936. *75.* 189.
NICOLAYSEN, R., Ibidem 1934. *69.* Suppl. 4.
NORMAN, A.-C. and B. Jensen, Z. anal. Chem. 1931. *83.* 113.
STOLBA, F., S. B. Böhm. Wiss. Prag 1876. 210.

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Untersuchungen über Dextran und sein Verhalten bei parenteraler Zufuhr. I.

Von

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Chemische und physikalisch-chemische Untersuchungen über Dextran.

Seit Einführung der Bluttransfusion als therapeutische Massnahme hat es sich gezeigt, dass sie mit praktischen Übelständen verbunden ist, auch in solchen Fällen, wo zur Erzielung eines therapeutischen Effektes nicht Gesamtblut erforderlich ist, sondern statt dessen Plasma verwendet werden kann. Man hat deshalb das Problem zu vereinfachen gesucht, indem man bequemer zugängliche Kolloide, in blutisotonischen Salzlösungen gelöst, als Infusionslösung einführte. Von diesen dürften die von BAYLISS 1917, HANZLIK und KARSNER, 1920, eingeführten Gummilösungen die bekanntesten sein. Sie wurden im Weltkriege 1914—1918 weitgehend angewendet und als »Gum saline« bezeichnet. Auch andere Substanzen wie Gelatine (BAYLISS 1917), Polyvinylalkohol (JORNS 1937, STIERLEN 1939, HUERPER 1939 a und b, und HUERPER, LANDSBERG und ESKRIDGE 1940), Pektin (HARTMAN und Mitarbeiter 1941), Polyvinylpyrrolidon (HECHT und WEESE 1943, KLEES 1943) u. s. w. sind zur Verwendung gekommen. Im Zusammenhange damit, dass sie körperfremde Substanzen sind, haften indessen allen diesen Nachteile an. Einige von ihnen haben antigene Eigenschaften, andere wirken dadurch schädlich, dass der Organismus sie nicht oder nur langsam abzubauen oder auszuscheiden imstande ist.

Wir wollen hier nicht auf die physiologischen Voraussetzungen der Anwendbarkeit einer Substanz für diese Zwecke eingehen, sondern nur betonen, dass sie in ihren physikalisch-chemischen und physiologischen Eigenschaften dem Bluteiweiss so weit wie möglich gleichen, sowie serologisch indifferent sein muss, und ferner, dass der Organismus sie in angemessener Zeit abzubauen und auszuscheiden vermag. Diese Fragen werden in einer folgenden Arbeit eingehender diskutiert.

Eine in dieser Hinsicht bisher nicht untersuchte Substanz bildet das unter der Bezeichnung Dextran bekannte neutrale Polysaccharid. Hier werden wir einige chemische und physikalisch-chemische Untersuchungen von Dextran mitteilen. In einer folgenden Mitteilung werden Hydrolyseprodukte von Dextran beschrieben, sowie ihre Eliminierung nach parenteraler Zufuhr und ihre Wirkung im experimentellen Schock.

Das Dextran wurde schon im 19. Jahrhundert entdeckt, und in dessen letzten Jahrzehnten enthält die Zuckerliteratur mehrere Abhandlungen, wo es als ein Stoff der Formel $(C_6H_{10}O_5)_n$ beschrieben wird, welcher bei saurer Hydrolyse lediglich Glukose liefert. Auch wurde die Bildung von Dextran in Zuckerrübensäften nachgewiesen, welche mit dem Bakterium Leuconostoc mesenteroides (oder Varianten von diesem unter verschiedenen Namen) infiziert waren. Wir können hier nicht alle älteren Arbeiten über Dextran anführen und begnügen uns mit dem Hinweis auf den Artikel über Dextran von Rümpler (1898) sowie auf Wohryzek (1928).

Während der letzten Jahrzehnte wurde eine Anzahl Dextranuntersuchungen durchgeführt, von denen wir hier nur einen Teil erwähnen können. (Tarr und Hibbert 1931, Fitzgerald 1933, Carruthers und Cooper 1936, Sacchetti 1936, Fowler und Mitarbeiter 1937, Stacey und Youd 1938, Peat und Mitarbeiter 1938, 1939, Daker und Stacey 1939, Hassid und Barker 1940).

Von den wichtigeren Ergebnissen dieser Studien seien folgende genannt:

Die Dextranbakterien kommen wahrscheinlich in mehreren Varianten vor, doch synthetisieren diese sämtlich Polysaccharide, die nur aus Glukose aufgebaut sind.

Durch Methylierung des Dextrans und nachfolgende Hydrolyse sowie Identifizierung der Hydrolyseprodukte hat sich zeigen lassen, dass das Polysaccharid aus Glukopyranoseeinheiten besteht, welche, besonders durch die Kohlenstoffatome Nr 1 und 6, zu

langen Ketten vereinigt sind. Spätere Resultate deuten auch auf eine verzweigte Kette (LEVI und MITARBEITER 1942). Durch »Endgruppenanalysen« hat man die Länge der Ketten festzustellen versucht und ist dabei zu ungleichen Ergebnissen für verschiedene Dextranprodukte gekommen.

Die beschriebenen Dextrane waren teils löslich, teils nicht löslich in Wasser. Die nicht wasserlöslichen sollen in z. B. 1-n NaOH löslich sein. Die spezifische Drehung der verschiedenen beschriebenen Dextranpräparate war wechselnd.

Um zuerst das Polysaccharid zu charakterisieren, das wir für die Darstellung der später (in Mitt. II) beschriebenen Infusionslösung verwendet haben, berichten wir hier über einige unserer Versuche mit Dextranlösung aus Zuckerrübenpressaft, welcher mit Leuconostoc mesenteroides infiziert worden war.

In einem Cellophansack wurde der Pressaft gegen fliessendes Wasser dialysiert und sodann das Eiweiss durch Zusatz von Citratpuffer von pH 3.4 ausgefällt. Nach Einengung der Lösung durch Verdunsten im Vakuum wurde abermals gegen Wasser dialysiert. Die einige Prozent Dextran enthaltende gewonnene Lösung war sehr viskos. Ausser dem weiter unten beschriebenen Dextran fanden sich in ihr auch kleinere Mengen anderer, sowohl neutraler wie saurer Polysaccharide sowie einiger Abbauprodukte von Eiweiss u. a. (INGELMAN und TISELIUS 1943). Aus dieser Lösung konnte das Dextran in sehr reinem Zustande durch wiederholtes Umfällen mit Alkohol gewonnen werden. Wurde nämlich zu der Lösung Alkohol gegeben (z. B. ihr doppeltes Volumen) so fiel das Dextran als fadenförmiger Niederschlag aus. Nach drei oder vier Umfällungen war die Substanz stickstoffrei und schien auch keine anderen Kohlehydrate mehr zu enthalten. Hiernach wurde die Lösung nochmals dialysiert; in einigen Fällen, wenn die Gewinnung salzfreier Proben erwünscht war, erfolgte auch Elektrodialyse.

Das reindargestellte Kohlehydrat war zu einigen Prozent in Wasser löslich; die erhaltenen Lösungen waren sehr zäh.

In Fig. 1 ist die relative Viskosität als Funktion der Dextrankonzentration aufgezeichnet. Die Messungen wurden bei + 23.0° C in Lösungen vorgenommen, welche an Na_2HPO_4 wie an NaH_2PO_4 je 0.025 molar waren. Die Konzentration wurde durch Eindampfen bei 110° C bestimmt.

Die Substanz sprach erwartungsgemäss auf die üblichen Koh-

lehydratreagentien wie z. B. Orcin-Schwefelsäure an. Mit Jod gaben die Lösungen keine Färbung. Die Substanz war frei von Stickstoff. Phosphorsäure konnte nicht nachgewiesen werden. Das Kohlehydrat enthielt keine leicht abspaltbaren Acetylgruppen. Pentosen oder Alduronsäuren waren nicht nachweisbar.

Das Polysaccharid wurde durch sechsstündiges Kochen mit vierprozentiger Schwefelsäure hydrolysiert. Aus dem Hydrolyseprodukt konnte Glukose-Phenylosazon dargestellt werden.

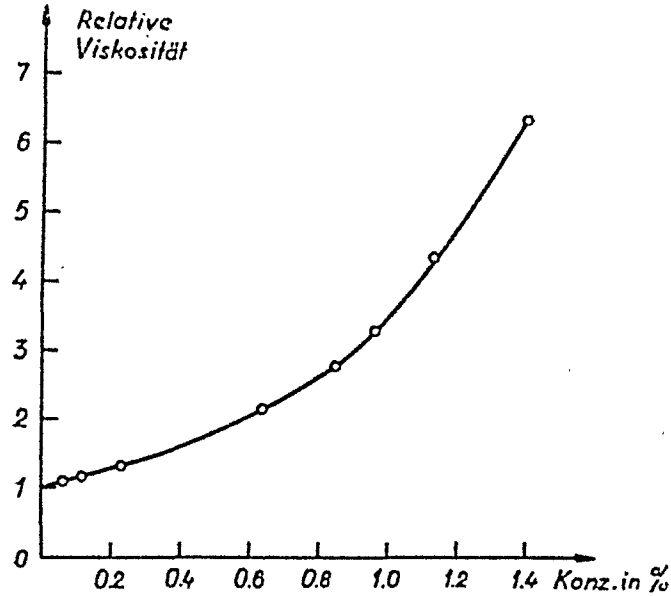

Fig. 1.

Die spezifische Drehung $[\alpha]_D^{20°}$ des Kohlehydrates ergab sich in wässriger Lösung zu $+ 195°$.

Ein quantitativ durchgeführter Hydrolysierungsversuch mit vierprozentiger Schwefelsäure zeigte, dass die spezifische Drehung sich nach sechsstündigem Kochen nicht mehr änderte. In Fig. 2 ist die spezifische Drehung als Funktion der Kochdauer aufgezeichnet. Der Berechnung der spezifischen Drehung liegt die ursprüngliche Konzentration des hochmolekularen Kohlehydrates zu Grunde (korrigiert für die geringen von der Verdunstung durch den Rückflusskühler verursachten Verluste). Erwartungsgemäss nimmt die Drehung ständig bis zu einem konstanten Werte ab.

Im Hydrolysate wurde nunmehr die Konzentration an reduzierendem Zucker mit Hilfe von HAGEDORN-JENSENS Titrationsmethode ermittelt. Wird dann die spezifische Drehung berechnet,

jedoch auf Grund der Monosaccharidkonzentration, so ergibt sich der Wert $+52.6°$, welcher mit dem der Glukose gut übereinstimmt.

Das Hydrolysat wurde auch in TISELIUS' Adsorptionsapparat (1940—41) untersucht, unter Verwendung von aktiver Kohle als Adsorbens. Das Adsorptionsdiagramm zeigte nur eine Kohlehydratkomponente. Es hatte genau dasselbe Aussehen wie das einer gleichstarken Glukoselösung.

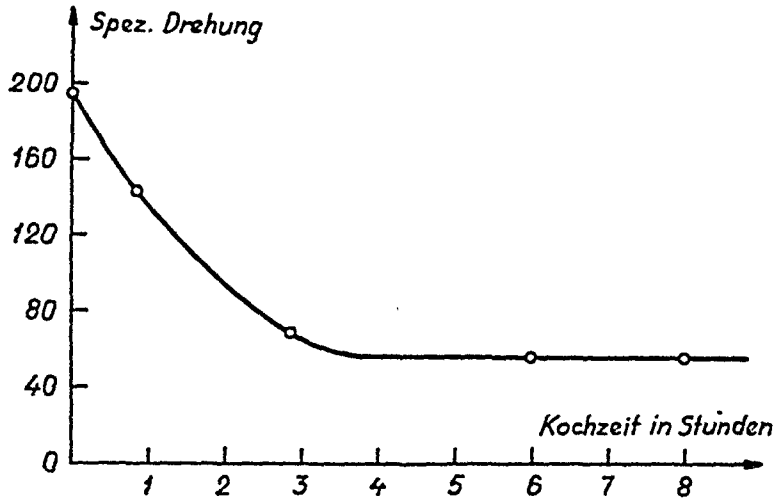

Fig. 2.

Wie bereits erwähnt, konnte aus einem Hydrolysate des Polysaccharides Glukose-Phenylosazon dargestellt werden. Da nun auch die Drehung des Hydrolysates mit der der Glukose gut übereinstimmt und ferner sein Adsorptionsdiagramm das gleiche ist wie das der Glukose, dürfte kein Zweifel darüber bestehen, dass das Polysaccharid aus Glukose aufgebaut ist.

An dem Polysaccharid wurde eine Bestimmung des Brechungsindexinkrementes vorgenommen. Dabei ergab sich die Grösse dn/dc zu $1.53 \cdot 10^{-3}$ für die gelbe (577/79 mμ) und zu $1.54 \cdot 10^{-3}$ für die grüne (546 mμ) Quecksilberlinie. (Die Messung wurde bei einer Konzentration von 1.44 % ausgeführt, welche durch Eintrocknen bei 110° C bestimmt wurde.)

Das partielle spezifische Volumen wurde in wässriger Lösung mit einer Dextrankonzentration von 1.31 % (durch Eintrocknen bei 110° C bestimmt) ermittelt, wobei der Wert 0.59 gefunden wurde. In der Literatur ist z. B. für Stärke der Wert 0.60 angegeben.

Bei der Elektrophorese wandert das Dextran sehr langsam, was ja der Erwartung entspricht, da es ein neutrales Polysaccharid ist. Bei einigen Messungen in Pufferlösungen von pH 6.8, die an Na_2HPO_4 sowie an NaH_2PO_4 0.025 molar waren, ergab sich eine Wanderungsgeschwindigkeit gegen die Pluselektrode von $0.5 \cdot 10^{-5}$ cm^2/sec. Volt, was für neutrale Kohlehydrate normal ist.

Bei der Adsorptionsanalyse einer Dextranlösung in TISELIUS' Adsorptionsapparat mit aktiver Kohle als Adsorbens konnte nur eine Kohlehydratkomponente wahrgenommen werden. Die Adsorption an der Aktivkohle war ziemlich schwach, das Adsorptionsdiagramm sah aus wie Fig. 3 zeigt. (Bezüglich Ausführung der Adsorptionsanalyse und Aufstellung des Diagramms verweisen wir auf (TISELIUS 1940, 1941 a och b). Der Gipfel rechts stammt von dem Phosphatpuffer her, der scharfe linke vom Dextran.)

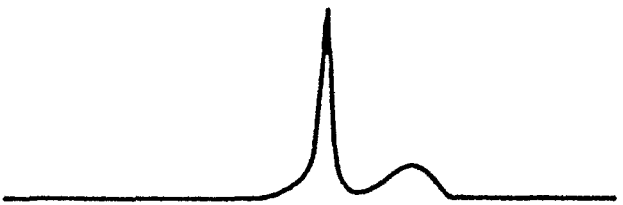

Fig. 3.

Fällt man Dextran mit Alkohol, so entsteht eine ungewöhnlich fadenartige Fällung. Schon danach darf man erwarten, dass das Polysaccharid aus Fadenmolekeln besteht. Auch aus verschiedenen physikalisch-chemischen Bestimmungen ging hervor, dass die Dextranmolekel wahrscheinlich langgestreckt ist.

Bei einem — von fil. lic. O. SNELLMAN ausgeführten — Versuch, die Molekellänge aus der Strömungsdoppelbrechung abzuleiten, ergab sich Uneinheitlichkeit der Molekellänge des Polysaccharides. (Für diese wurde ein wegen der Uneinheitlichkeit äusserst unsicherer Wert von einigen tausend Å. E. gefunden.)[1]

Es wurden einige Ultrazentrifugierungsversuche mit SVEDBERGS Ölturbinenzentrifuge an verschiedenen Polysaccharidkonzentrationen angestellt. Beobachtet wurde nach LAMMS Skalenmethode (1937); die Messungen wurden in Phosphatpuffern vorgenommen, die an Na_2HPO_4 sowohl wie an NaH_2PO_4 0.025

[1] Noch nicht abgeschlossene Untersuchungen von B. INGELMAN und K. SIEGBAHN mit dem Elektronenmikroskop im Forskningsinstitutet för Fysik, Stockholm, haben obenfalls eine fadenförmige Struktur des Dextrans gezeigt. Diese Untersuchungen werden später an anderer Stelle veröffentlicht.

molar waren. Dabei ergab sich eine sehr beträchtliche Abhängigkeit der Sedimentationskonstanten von der Konzentration, wie sie für Molekeln von langgestreckter Form gewöhnlich ist. (Fig. 4)

Im Konzentrationsgebiete 0.4—1.4 % wurden einfache Sedimentationsdiagramme mit einem Gipfel gefunden. (Bezüglich Ausführung der Zentrifugierungen und Aufstellung der Zentrifugierungsdiagramme etc. sei auf SVEDBERG und PEDERSEN (1940) verwiesen. Hier sei nur betont, dass jeder Gipfel im Zentrifugierungsdiagramm einem hochmolekularen Stoffe entspricht.)

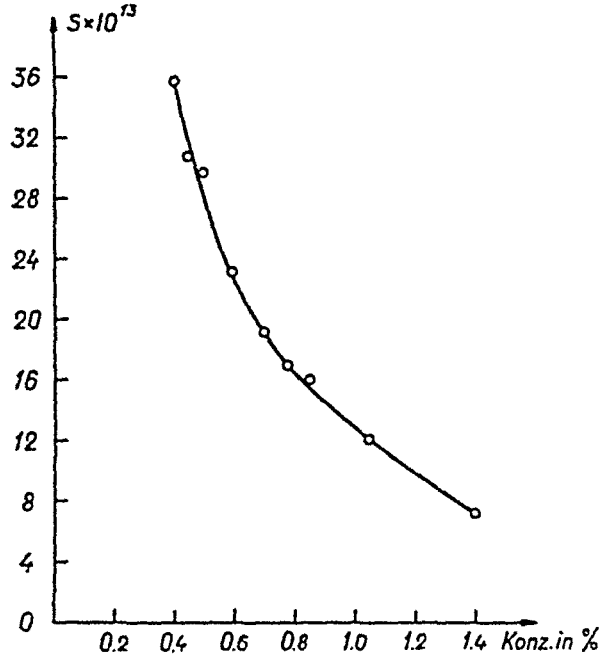

Fig. 4.

Unterhalb 0.4 % traten dagegen statt eines einheitlichen Gipfels in den Diagrammen eine Menge kleinerer Gipfel auf, für welche keine sicheren Sedimentationskonstanten berechnet werden konnten.

Extrapolation der Sedimentationskonstanten auf die Konzentration Null konnte nicht vorgenommen werden.

Fig. 5 zeigt das Aussehen der Zentrifugierungsdiagramme für einige verschiedene Dextrankonzentrationen. Z bezeichnet die Skalenstrichverschiebung und X den Abstand von der Rotationsaxe (SVEDBERG 1940). Die Zentrifuge lief mit 59 000 U. p. M., entsprechend einer Zentrifugalkraft der 250 000-fachen Erdschwere.

Ferner wurden auch einige Diffusionsversuche ausgeführt (nach LAMM, 1937), wobei die Diffusionskonstanten für verschiedene Konzentrationen ermittelt wurden (Tabelle 1). In einem Versuche ergab sich eine extrem niedrige Diffusionskonstante. Berechnet wurde die Diffusionskonstante nach der Formel $D_A = \frac{A^2}{4\pi t\, H_{max}^2}$, wo t die Diffusionszeit, A die Fläche unter der Diffusionskurve und H_{max} die Maximalordinate der Kurve bedeutet. Die Versuchstemperatur betrug 20.0° C. Die Diffusionsversuche wiesen darauf hin, dass das Dextran nicht vollkommen monodispers sein konnte.

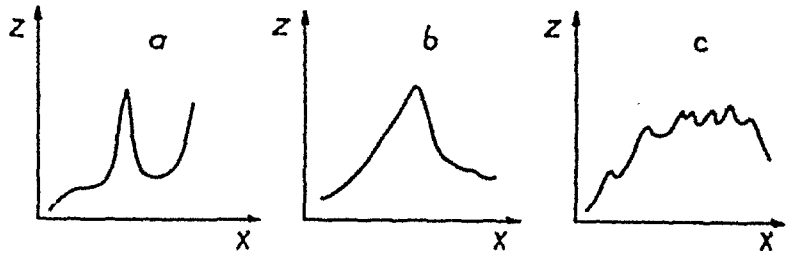

Fig. 5.

Sedimentationsdiagramme von Dextranlösungen verschiedener Konzentration.

a) 0.70 % Dextran 15 Min. nach Erreichung der vollen Geschwindigkeit.
b) 0.40 % » 7 » » » » » »
c) 0.20 % » 7 » » » » » »

Da die Molekulargrösse anscheinend stark variiert, lässt sich eine genaue Angabe für das Molekulargewicht des Dextranes nicht machen. Die Zentrifugierungsdiagramme von Lösungen mit Dextrankonzentrationen unter 0.35 % zeigten nämlich statt eines einheitlichen Gipfels mehrere verbreiterte Gipfel. Bei fil. lic. O. SNELLMANS Untersuchung mittels Strömungsdoppelbrechung ergab sich auch Uneinheitlichkeit der Länge der Dextranmolekeln. Doch wurde bei höheren Dextrankonzentrationen trotz der anscheinend recht erheblichen Variabilität des Molekulargewichtes der Dextranmolekeln ein einziger scharfer Gipfel im Zentrifugierungsdiagramm gefunden. Dies dürfte seinen Grund in der fadenähnlichen Gestalt der Dextranmolekeln haben, da fadenförmige Molekeln ihre Sedimentation gegenseitig beeinflussen.

Setzt man die Werte von Sedimentations- resp. Diffusionskonstante für etwa 0.4 % (Extrapolation der Sedimentationskonstanten auf die Konzentration Null war untunlich) in SVEDBERGS Formel für die Berechnung des Molekulargewichtes ein, so ergeben

sich sehr hohe Werte. Aus einer Sedimentationskonstanten $36 \cdot 10^{-13}$, einer Diffusionskonstanten $0.27 \cdot 10^{-7}$ und einem partiellen spezifischen Volumen 0.59 erhält man das Molekulargewicht mit 8.10^6. Dieser Wert darf jedoch nicht als exakter Ausdruck der Molekelgrösse angesehen werden, denn wir konnten die Konstanten nicht auf die Konzentration Null extrapolieren und alles weist darauf hin, dass die Molekulargrösse stark wechselt. Die vorgenommene Berechnung zeigt indessen, dass das Dextran ein sehr hohes Molekulargewicht hat. (Jedenfalls war dieses sehr hoch für das hier untersuchte Dextran. Das Molekulargewicht kann ja möglicherweise mit der Infektionsdauer des Saftes weitgehend veränderlich sein.)

Tab. 1.

Polysaccharidkonzentration in %	Diffusionskonstante $D_A \cdot 10^7$
0.70	0.45
0.50	0.52
0.35	0.27
0.35	0.29
0.35	(0.07—0.13)
0.25	0.40

Mit der Absicht der Gewinnung eines Antiserums wurden zwei Kaninchen jeden fünften Tag 2 ml 0.5 prozentiger Dextranlösung eingespritzt; diese Einspritzungen wurden zwei Monate lang fortgesetzt. Hierauf wurde das Serum mit verschiedenen Mengen Dextranlösung geprüft und gefunden, dass kein Präzipitat erhalten werden konnte. Nach allem zu schliessen hatte Präzipitinbildung ersichtlich nicht stattgefunden.[1]

Zusammenfassung.

Früher sind verschiedene hochmolekulare Stoffe als therapeutisches Substitut für die normalen Serumproteine vorgeschlagen worden. Eine Substanz, die mit Rücksicht auf ihre Anwendbarkeit als solches noch nicht untersucht worden ist, ist das neutrale Polysaccharid Dextran.

Nach einer kurzen Wiedergabe unserer wichtigsten Kenntnisse über das Dextran werden eigene chemische und physikalisch-chemische Untersuchungen über diesen Stoff mitgeteilt, sowie

[1] Diese Untersuchung über die Antikörperbildung verdanken wir Herrn Dozent S. GARD.

seine Darstellung aus Zuckerrübensaft, der mit Leuconostoc mesenteroides infiziert war, beschrieben. Durch Dialyse und Fällung und wiederholte Umfällung mit Alkohol wird ein reines stickstoffreies Dextran erhalten. Die Analyse dieses Produktes zeigt, dass es einzig aus Glukose aufgebaut ist. Physikalisch-chemische Untersuchungen ergeben eine sehr hohe Viskosität, in Phosphatpuffer bei pH 6.8 eine sehr langsame elektrophoretische Wanderung zur Pluselektrode, sowie eine spezifische Drehung $[\alpha]_D^{20^\circ} = +195^\circ$. Bei der Adsorptionsanalyse wird nur eine Kohlehydratkomponente beobachtet. Das Dextranmolekül ist nach allem zu urteilen langgestreckt, doch von nicht einheitlicher Molekularlänge. Die Bestimmung der Sedimentationskonstanten, der Diffusionskonstanten und des partiellen spezifischen Volumens zeigt, dass das Molekulargewicht sehr hoch ist, von der Grössenordnung viele Millionen. Ein exaktes Molekulargewicht kann wegen der uneinheitlichen Molekulargrössen nicht angegeben werden.

Das hier vorliegende Dextran ruft keine Präzipitinbildung hervor.

Die Verfasser möchten Herrn Professor ARNE TISELIUS für alle guten Ratschläge bei der Ausführung dieser Untersuchungen besonders danken. Die Arbeit ist in den Laboratorien der Professoren THE SVEDBERG und ARNE TISELIUS am Physikalisch-chemischen Institut in Upsala ausgeführt worden. Svenska Sockerfabriks A.-B. hat durch finanzielle Hilfe die Durchführung der Arbeit unterstützt.

Literatur.

BAYLISS, W. M., Proc. Roy. Soc. Ser. B. 1917. *89.* 380.
CARRUTHERS, A., and E. A. COOPER, Biochem. J. 1936. *30.* 1001.
DAKER, W. D., and M. STACEY, J. chem. Soc. 1939: *1.* 585.
FITZGERALD, J. G., Trans. Roy. Soc. Can. 1933. *27.* 1.
FOWLER, F., I. BUCKLAND, F. BRAUNS and H. HIBBERT, Canad. J. Res. Sect. B. 1937. *15.* 486.
HANZLIK, P., and H. KARSNER, J. Pharmacol. 1920. *14.* 379.
HARTMAN, T. W., V. SCHELLING, H. N. HARKINS and B. BURSH, Ann Surg. 1941. *114.* 212.
HASSID, W. Z. and H. A. BARKER, J. Biol. Chem. 1940. *134.* 163.
HECHT, G. and H. WEESE, Münch. med. Wschr. 1943. *90.* 11.
HUERPER, W. C., Amer. J. Path. 1939. *15.* 644.
—, Arch. Path. 1939. *28.* 510.
—, J. W. Landsberg and L. C. Eskridge, J. Pharmacol. 1940. *70.* 201.

INGELMAN, B. and A. TISELIUS, Förhandlingar vid Svenska Sockerfabriksdirigenternas Förenings sammanträden 1943. Del 2.
JORNS, G., Ther. Gegenwart 1937. *78.* 394.
KLEES, E., Münch. med. Wschr. 1943. *90.* 29.
LAMM, O., Nova Acta Reg. Soc. Sci. Upsaliensis. 1937. *10.* Ser IV. no. 6.
LEVI, I., L. HAWKINS and H. HIBBERT, J. Amer. Chem. Soc. 1942. *64.* 1959.
PEAT, S., M. STACEY and E. SCHLÜCHTERER, Nature 1938. *141.* 876.
PEAT, S., M. STACEY and E. SCHLÜCHTERER, J. chem. Soc. 1939: 1. 581.
RÜMPLER, A., Die Nichtzuckerstoffe der Rüben, Braunschweig 1898.
SACCHETTI, M., Zbl. Bakteriol. Parasitenkunde, Impfkrankheiten 1936. II. 95. 102.
STACEY, M. and F. R. YOUD, Biochem. J. 1938. *32.* 1943.
STIERLEN, G., Z. ges. exp. Med. 1939. *106.* 201.
SVEDBERG, T., and K. O. PEDERSEN, Die Ultrazentrifuge, Dresden und Leipzig 1940.
TARR, H. L. A., and H. HIPPERT, Canad. J. Res. 1931. *5.* 414.
TISELIUS, A., Ark. f. Kemi 1940. 14 B. no. 22. 1941. 14 B. no. 32. 1941. 15 B. no. 6.
WOHRYZEK, O., Chemie der Zuckerindustrie, Berlin 1928.

Aus dem Medizinisch-chemischen Institut der Universität Helsinki.

Über die Einwirkung der Entfernung der Nebennieren auf die Phosphorylierung im Muskel.

Von

O. HELVE.

Eingereicht am 22. November 1943.

Zur Erklärung der Wirkungsweise der Nebennierenrinde haben VERZÁR und LASZT (1935, 1936, 1937) die Rindenschicht in engem Zusammenhang mit Phosphorylierungserscheinungen gebracht. Demnach bewirkt die Entfernung der Nebennieren durch eine Störung der Phosphorylierung eine Herabsetzung der Kohlenhydrat- und Fettresorption sowie der Bildung der Lactoflavinphosphorsäure aus Lactoflavin.

Bei der Durchführung meiner systematischen Untersuchungen über den Stoffwechsel der Nebennieren (HELVE, 1937, 1940) erschien es daher begründet, auch die Phosphorylierungserscheinungen im Muskel bei Mangel an Rindehormon zu behandeln, insbesonders da über diese Frage in der Literatur keine früheren Angaben vorlagen. Wegen der im Muskel bei nebennierenlosen Tieren auftretenden Adynamie erschien der Gedanke einer Störung der Phosphorylierung im Muskel durchaus möglich. Diese Untersuchungen, bei denen die Phosphorylierung des in Phosphatlösung gepufferten Muskelbreis von nebennierenlosen und Kontrollratten bei Gegenwart von Natriumfluorid und Glykogen verglichen wurde, ergaben jedoch keine Störungen der Phosphorylierung.

Gegen die Phosphorylierungstheorie spricht auch eine Reihe anderer Untersuchungen.

Nach den Feststellungen von DEUEL, HALLMAN, MURRAY und SAMUELS (1937), ALTHAUSEN, ANDERSON und STOCKHOLM (1939)

sowie BARNES, WICK, MILLER und MACKAY (1939) ist die Störung der Fett- und Kohlenhydratresorption bei nebennierenlosen Versuchstieren auf den Schockzustand und Flüssigkeitsverlust und nicht direkt auf den Mangel an Rindehormonen zurückzuführen. Sie haben bei ihren Versuchen beobachtet, dass keine Resorptionsstörung der erwähnten Art auftritt, wenn nur die Versuchstiere vor dem cirkulatorischen Kollaps geschützt werden können. Nach FERREBEE (1940) ist die Phosphorylierung des B_1-Vitamins sowie des Lactoflavins bei nebennierenlosen Versuchstieren normal. OCHOA und ROSSITER (1940) kamen in Bezug auf Vitamin B_1 zu dem gleichen Ergebnis. Nach NELSON (1940) ist es nicht möglich durch Verabreichung von phosphoryliertem Lactoflavin die Lebenszeit nebennierenloser Tiere zu verlängern.

Andererseits ist zu bemerken, dass nach den Untersuchungen von SCHUMANN (1940) die Phosphorylierung im Muskel nebennierenloser Ratten deutlich schwächer wäre als normal. Ebenso behaupten VERZÁR und MONTIGEL (1942) auf Grund ihrer kürzlich durchgeführten Untersuchungen, dass die Phosphorylierung im Muskel nebennierenloser Ratten viel langsamer sei als die der Kontrolltiere. Die Tatsache, dass ich zu anderen Ergebnissen kam, erklären sie damit, dass ich eine zu lange Bebrütungszeit ($3^1/_2$ Stunden bei 37°) gebraucht habe.

Später habe ich mich mit der gleichen Frage von neuem beschäftigt und behandle nachstehend Versuche, in welchen vergleichsweise sowohl die von SCHUMANN als auch die von VERZÁR und MONTIGEL gebrauchten Versuchsverhältnisse angewandt wurden.

In den zuerst behandelten Versuchsreihen wurde untersucht, welche Wirkung möglicherweise das Natriumbikarbonat hat, welches SCHUMANN bei seinen Versuchen in der Inkubationsflüssigkeit verwendet hat.

Zu der Versuchsreihe gehörten 10 Ratten, deren Nebennieren extirpiert waren, sowie 10 Kontrolltiere. Das Verfahren war im übrigen folgendes:

2 g feingemahlener Muskelbrei wurde mit 6 ml Lösung gemischt, welche in Bezug auf Natriumbikarbonat 0.025 mol. und auf Natriumfluorid 0.5 % war, sowie 10 mg Glykogen enthielt. Die Muskelbreiflüssigkeitsmischung wurde in einem kleinen Erlenmeyerkolben nach Hineinleitung von Sauerstoff im Wasserthermostat bei 37° geschüttelt. Nach zwei Stunden wurde das Eiweiss mit 2 ml 20 % Trichloressigsäure ausgefällt und dann nach Filtrierung der Phosphor nach LOHMANN und JEDRASSIK (1926, 1928) bestimmt. Gleichzeitig wurde ein Versuch

ausgeführt, bei dem der Phosphor sofort bestimmt wurde. Aus der Differenz der Phosphorwerte geht die Menge des esterifizierten Phosphors hervor.

Die Versuche wurden 4—8 Tage nach der Operation durchgeführt, als die Tiere adynamisch geworden waren und an Gewicht erheblich abgenommen hatten.

Die Ergebnisse gehen aus folgenden Tabellen hervor:

Tabelle I.

Phosphorylierungsversuche mit Muskel der Kontrollratten. (P in mg%.)

Nr.	Geschlecht	Gewicht (g)	2 g Muskel, 10 mg Glykogen 5 ccm 0.025 mol. Na-bikarbonat-Lös., 1 ccm 3 %-ige NaF-Lös.		Esterifizierter Phosphor	
			Phosphor sofort bestimmt	2 Std. bei 37°. Dann Phosphorbestimmung	in mg	in %
1	♂	175	64.9	42.1	22.8	35
2	♂	189	67.7	47.1	20.6	30
3	♂	215	65.1	44.3	20.8	32
4	♂	245	80.6	53.9	26.7	33
5	♂	270	96.9	66.7	30.2	31
6	♂	255	88.7	55.0	33.7	38
7	♂	226	73.3	39.0	34.3	47
8	♂	290	92.1	65.6	26.5	29
9	♂	315	72.9	44.8	28.1	39
10	♀	232	83.8	56.0	27.8	33
		Mittelwerte	78.6	51.5	27.2	35

Wie aus den Tabellen hervorgeht, schwankte die Menge des bei den Phosphorylierungsversuchen der Kontrollratten esterifizierten Phosphors zwischen 30—47 %, wobei der Mittelwert 35 % betrug. Der entsprechende Wert der nebennierenlosen Versuchstiere war mit Ausnahme von zwei Fällen etwas grösser, bei einem Mittelwert von 44 %.

Mit der von mir angewandten Methode konnte also festgestellt werden, dass im Muskelbrei der nebennierenlosen Ratten bei Gegenwart von Natriumbikarbonat, Glykogen und Natriumfluorid in den meisten Fällen eine sogar etwas stärkere Esterifizierung stattfindet als bei den Kontrollratten. Die Versuchsergebnisse gehen also eher in umgekehrter Richtung als das, was SCHUMANN bei seinen Untersuchungen festgestellt hat und geben keine

Tabelle II.

Phosphorylierungsversuche mit Muskel der nebennierenlosen Ratten. (P in mg%).

Nr.	Geschlecht	Lebensdauer nach der Operation (Tage)	Gewichtsabnahme (g)	2 g Muskel, 10 mg Glykogen 5 ccm 0.025 mol. Na-bikarbonat-Lös., 1 ccm 3%-ige NaF-Lös.		Esterifizierter Phosphor	
				Phosphor sofort bestimmt	2 Std. bei 37°. Dann Phosphorbestimmung	in mg	in %
11	♂	5	186—168	62.4	32.0	30.4	49
12	♂	6	210—180	79.1	43.4	35.7	45
13	♂	8	211—182	85.2	47.3	37.9	44
14	♂	5	257—239	68.1	51.8	16.3	24
15	♂	5	252—234	71.8	32.2	39.6	55
16	♂	7	255—230	68.1	33.6	34.5	51
17	♀	4	175—162	75.9	34.3	41.6	55
18	♂	7	271—230	83.2	45.3	37.9	46
19	♂	8	253—220	91.9	52.3	39.6	48
20	♂	7	261—244	84.1	57.1	27.0	32
			Mittelwerte	77.0	42.9	34.1	44

Bestätigung für die Theorie von VERZÁR und LAZST, deren Richtigkeit SCHUMANN als Erster bewiesen haben will.

In den folgenden Versuchsreihen wurde der mögliche Einfluss der Zeit auf die Phosphorylierung untersucht.

Für die Versuche wurden 10 nebennierenlose Ratten verwendet sowie 5 Kontrolltiere, bei denen eine Scheinoperation gemacht wurde, bei welcher die Nebennieren nicht entfernt wurden. Die Versuche wurden 5—10 Tage nach der Operation durchgeführt. Die nebennierenlosen Versuchstiere wurden getötet, sobald Zeichen von Adynamie an ihnen bemerkbar waren.

Die Phosphorylierungsversuche führte ich auf zwei Arten aus:

a) 1 g im Latapie verkleinerter Muskel, 1 ml 1 % $Na_2HPO_4 \cdot 2\ H_2O$-Lösung, 1 ml 2 % NaF-Lösung sowie 30 mg Glykogen. Das Eiweiss wurde mit 7 ml 7 % Trichloressigsäure ausgefällt (Tabelle III).

b) 0.5 g gleicher Muskelbrei, 0.5 ml 2 % $NaHCO_3$-Lösung, 0.5 ml $3^1/_2$ % NaF-Lösung sowie 1 ml 0.5 % Glykogenlösung. Das Eiweiss wurde mit 8 ml 7 % Trichloressigsäure ausgefällt (Tabelle IV).

Der Phosphor des eiweissfreien Filtrats wurde nach 20, 60 und 210 Minuten dauernder Bebrütung im Wärmeschrank (37°) bestimmt. Ausserdem wurde die Phosphorbestimmung sofort, ohne Bebrütung durchgeführt (0-Minutenwerte in den Tabellen).

Die auf beide Arten (a und b) durchgeführten Versuche zeigen, dass die Phosphorylierung ihren Maximalwert schon nach 20 Minuten erreicht (Tabellen III und IV). (Einige 10 und 30 Minuten nach der Behandlung durchgeführte Phosphorbestimmungen stützen diese Beobachtung.)

Tabelle III.

Muskelphosphorylierungsversuche mit Phosphatpuffer. (P in mg%).

Nr.	Versuchsdauer Tage	Gewichtsveränderung (g)	In 37°				Esterif. P (20 Min.)	
			0 Min.	20 Min.	60 Min.	210 Min.	mg	%
			Scheinoperierte Ratten:					
1	5	275—286	321.1	150.0	153.2	157.6	171.1	53
2	8	242—245	279.0	143.2	136.5	139.3	135.8	49
3	7	331—335	250.4	83.3	84.1	85.9	167.1	67
4	10	250—257	261.5	119.7	118.0	117.2	141.8	54
5	8	335—327	294.8	117.9	119.4	121.5	176.4	60
		Mittelwerte:	281.3	122.8	122.2	121.3	158.4	57
			Nebennierenlose Ratten:					
6	6	266—244	285.2	100.4	101.5	99.7	185.6	65
7	7	285—265	289.2	84.2	86.4	87.1	205.0	71
8	6	288—274	281.8	73.7	77.6	78.3	208.1	74
9	7	304—309	297.6	100.8	105.7	107.9	196.8	66
10	7	300—259	307.7	127.1	129.0	129.7	180.6	59
11	6	329—296	260.9	107.2	108.0	108.1	153.7	59
12	7	387—336	273.6	85.4	83.8	85.0	188.2	69
13	5	329—311	266.7	89.0	90.6	90.7	177.7	67
14	8	365—345	267.0	97.6	98.4	98.9	169.4	63
15	6	252—227	257.4	75.9	76.3	77.1	181.5	71
		Mittelwerte:	278.8	91.1	95.5	95.8	184.7	66

In den Versuchsreihen, bei welchen Phosphatpuffer gebraucht wurde (Tab. III), betrug der esterifizierte Phosphor der nebennierenlosen Ratten durchschnittlich 66 %, bei den Kontrollratten 57 %.

Bei Verwendung von Natriumbikarbonat anstelle von Phosphat (Tab. IV) war die Phosphorylierung im allgemeinen noch etwas stärker. Bei den nebennierenlosen Tieren erreichte sie durch-

Tabelle IV.

Muskelphosphorylierungsversuche mit Natriumbikarbonat. (P in mg%).

Nr.	In 37°				Esterif. P (20 Min.)	
	0 Min.	20 Min.	60 min.	210 Min.	mg	%
			Scheinoperierte Ratten:			
2	95.5	41.5	39.6	33.2	54.0	57
3	97.1	21.7	24.1	25.3	75.4	78
4	105.7	39.3	34.5	35.1	66.4	63
5	110.3	36.3	33.4	32.1	74.0	67
Mittelwerte:	102.2	34.7	32.9	31.4	67.5	66
			Nebennierenlose Ratten:			
8	109.9	19.2	22.3	23.2	90.7	83
9	130.8	25.2	27.9	28.7	105.6	80
10	112.3	36.3	38.7	39.4	76.0	68
11	104.9	30.5	30.3	32.1	74.4	71
12	100.4	27.1	28.0	30.3	73.3	73
13	97.1	21.7	22.4	23.5	75.4	78
14	117.7	31.8	31.0	30.4	85.9	73
15	97.2	27.3	25.9	25.2	69.9	72
Mittelwerte:	108.8	27.4	28.3	29.1	81.4	75

schnittlich einen Esterifizierungswert von 75 % und bei den Kontrolltieren 66 %.

Die Versuche zeigen, dass VERZÁRS und MONTIGELS Beobachtungen über die schnelle Erreichung des Maximums richtig sind.

Dagegen konnte langsamere oder geringere Phosphorylierung bei den nebennierenlosen Ratten — wie sie behauptet hatten — nicht festgestellt werden. Es ist schwer zu sagen, worauf der Unterschied der Untersuchungsergebnisse beruht. In der Diät der Versuchstiere gab es offensichtlich gewisse Unterschiede, was aber kaum entscheidend wirken dürfte, da die Abnahme des Gewichts, das Auftreten von Adynamie und die Zunahme des Kaliumgehaltes im Serum der operierten nebennierenlosen Tiere auf eine deutliche Rindenschichtinsuffizienz hinweisen.

Zusammenfassung.

Vergleichende Phosphorylierungsversuche zwischen normalen und nebennierenlosen Ratten werden beschrieben. Die Phosphorylierung im Muskel erreicht bei Gegenwart von Glykogen und Natriumfluorid in beiden Versuchstiergruppen das Maximum verhältnismässig schnell. Irgendwelche wesentlichen Unterschiede zwischen normalen und nebennierenlosen Tieren konnten weder bei kurzer noch bei langer Phosphorylierungszeit festgestellt werden.

Literatur.

ALTHAUSEN, T. L., E. ANDERSON und M. STOCKHOLM, Proc. Soc. exp. Biol. n. y. 1939, *40*, 342.
BARNES, R. H., A. N. WICK, E. S. MILLER und E. M. MACKAY, Ebenda 1939, *40*, 651.
DEUEL, H. J., JR, L. F. HALLMAN, S. MURRAY und L. F. SAMUELS, J. Biol. Chem. 1937, *119*, 607.
FERREBEE, J. W., Ebenda 1940, *136*, 719.
HELVE, O., Suomen Kemistilehti B 1937, *10*, 2.
—, Biochem. Z. 1940, *306*, 343.
LASZT, L. und F. VERZÁR, Ebenda 1937, *292*, 159.
LASZT, L. und F. VERZÁR, Pflüg. Arch. ges. Physiol. 1937, *239*, 653.
LOHMANN, K. und L. JENDRASSIK, Biochem. Z. 1926, *178*, 419.
LOHMANN, K. und L. JENDRASSIK, Ebenda 1928, *202*, 466.
NELSON, D., Amer. J. Physiol. 1940, *129*, 429.
OCHOA, S. und R. J. ROSSITER, J. Physiol. 1940, *97*, 1.
SCHUMANN, H., Pflüg. Arch. ges. Phys. 1940, *243*, 686.
VERZÁR, F. und L. LASZT, Biochem. Z. 1935, *276*, 11.
VERZÁR, F. und L. LASZT, Ebenda 1935, *278*, 396.
VERZÁR, F. und H. MONTIGEL, Helvet. chem. Acta 1942, *25*, 9.

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Über den Umsatz der Brenztraubensäure, α-Ketoglutarsäure und Citronensäure bei B-Avitaminosen.

Von

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Eingereicht am 22. November 1943.

Die Frage, ob sich im Mangel an Komponenten der B-Vitamingruppe Störungen im Stoffwechsel von α-Ketosäuren nachweisen lassen, ist in diesem Laboratorium seit 1934 Gegenstand von Untersuchungen gewesen. Es wurde dabei schon bald festgestellt, dass bei B-Vitaminmangelzuständen eine deutliche Anhäufung von Ketosäuren im Organismus nachzuweisen ist. Die ersten Untersuchungen schienen darauf hinzuweisen, dass es sich bei Vermehrung des Ketosäuregehaltes im Blut und Harn in erster Linie um eine Anhäufung der Brenztraubensäure handelte. Bei eingehenderen Untersuchungen wurde aber hinsichtlich des Vorkommens von Ketosäuren im Harn festgestellt, dass die Zunahme des Ketosäuregehaltes in Harn vielmehr auf eine vermehrte Ausscheidung der α-Ketoglutarsäure zurückzuführen war (SIMOLA, 1936 a, b, c), einer α-Ketosäure, über deren Vorkommen im Harn und in den Körperflüssigkeiten keine früheren Angaben vorlagen.

Eine Anhäufung der Acetonkörper war bei B-Avitaminosen dagegen nicht festzustellen.

Bei Entstehung der Störung des α-Ketosäurestoffwechsels schien dem Mangel des B_1-Vitamins die Hauptrolle zuzukommen.

Die Beobachtungen stimmten jedenfalls gut mit den Resultaten einer früheren Untersuchung überein, wobei ich auf Grund experimenteller Befunde die Aufmerksamkeit darauf richtete

dass die Co-Carboxylase ein zu der B-Vitamingruppe gehöriger Faktor sei (SIMOLA, 1932).

Um näheren Aufschluss über die Störung des α-Ketosäurestoffwechsels bei B_1-Vitaminmangel zu erhalten, wurden etwas später im Anschluss an diese Befunde Untersuchungen über den α-Ketosäureumsatz auch bei normalen Tieren vorgenommen. Es war denkbar, dass die vermehrte Ausscheidung der α-Ketoglutarsäure irgendwie auf eine primäre Erhöhung des Brenztraubensäuregehaltes im Blut zurückzuführen sei, und es gelang in der Tat, durch Verabreichung von Brenztraubensäure bei Versuchstieren sowie beim Menschen eine erhöhte Ausscheidung von α-Ketoglutarsäure hervorzurufen (SIMOLA, 1937, 1938 a; SIMOLA und KRUSIUS, 1939). Die Sache wurde aber dadurch verwickelter, dass es sich dann auch ergab, dass es möglich ist, durch Natriumsalze anderer Säuren sowie in gewissem Grade nur mit Alkali eine Erhöhung des α-Ketoglutarsäuregehaltes zu bewirken (SIMOLA und KRUSIUS, 1933, 1939; KRUSIUS, 1940).

Im Zusammenhang mit diesen Untersuchungen mit Normaltieren wurden auch weitere Versuche mit B-avitaminotischen Tieren vorgenommen. Es war die Absicht, mittels quantitativer Methoden Aufschluss über das Vorkommen der Brenztraubensäure und α-Ketoglutarsäure im Blut, in den Geweben und im Harn zu erhalten. Weiterhin war die Absicht, die Frage über die Anhäufung anderer α-Ketosäuren bei B-Avitaminosen, insbesondere bei Karenz des B_1-Vitamins, zu beleuchten und die Wirkung des Mangels an B_1-Vitamin auf den Umsatz des Alanins und der Glutaminsäure näher zu untersuchen.

In Anbetracht der neuen Befunde über die Beziehung der Citronensäure zur Brenztraubensäure wurde auch auf das Verhalten der Citronensäure bei Belastung mit Brenztraubensäure und verschiedenen anderen Säuren — u. a. mit C_4-Dicarbonsäuren, β-Oxybuttersäure und Acetessigsäure — sowie bei B-Mangelzuständen geachtet.

Es wurde dabei festgestellt, dass bei einer Erhöhung des Gehaltes an α-Ketoglutarsäure im Harn fast regelmässig gleichzeitig eine Steigerung der Citronensäureausscheidung vorkommt (SIMOLA, 1938 a, b; SIMOLA, KRUSIUS und ALAPEUSO, 1938; SIMOLA und KOSUNEN, 1939; SIMOLA und KRUSIUS, 1939; KRUSIUS, 1940).

Hinsichtlich der Wirkung der B-Vitaminkomponenten auf den Citronensäurestoffwechsel wurden bei B-Mangelzuständen je nach

dem Stadium des Mangelzustandes im Citronensäuregehalt des Rattenharns verschiedene Werte gefunden, wie schon früher vorläufig mitgeteilt wurde (KRUSIUS und SIMOLA, 1938; KRUSIUS, 1940).

Ausser Blut und Harn wurden als Untersuchungsmaterial verschiedene Gewebsarten verwendet. Bei B-avitaminotischen Tieren wandten sich diese Versuche zunächst dem Auftreten und dem Umsatz der Brenztraubensäure und der α-Ketoglutarsäure sowie der Citronensäure in den verschiedenen Organen zu.

Die vorliegende Arbeit beschränkt sich darauf, eine Zusammenfassung aus einer Anzahl von Versuchsreihen wiederzugeben, worin als unmittelbare Ergänzung meiner früheren, die Beziehungen zwischen dem α-Ketoglutarsäureumsatz und der B-Vitamingruppe betreffenden Untersuchungen versucht wird, durch quantitative Methoden das Auftreten von Brenztraubensäure und α-Ketoglutarsäure im Harn und im Blut bei B-Vitaminmangelzuständen zu beleuchten, während gleichzeitig auch dem Umsatz der Citronensäure Aufmerksamkeit gewidmet wird.

Es war die Absicht, die vorliegenden Untersuchungen, welche zum grossen Teil früher in Friedenszeiten durchgeführt wurden und damals wegen Schwierigkeiten in der Beschaffung von B-Vitaminpräparaten und des Misslingens einiger Tierversuchsreihen unvollständig blieben, später auf andere Weise zu ergränzen. Die besonderen Verhältnisse boten hierzu später jedoch nur begrenzte Möglichkeiten.

Zur Methodik.

Vom Standpunkt der Methodik aus verursachte besonders die quantitative Bestimmung der Brenztraubensäure und α-Ketoglutarsäure grosse Schwierigkeiten. Dies galt besonders für die Blutanalysen, bei denen sehr kleine Mengen dieser Substanzen bestimmt werden mussten. Für die Brenztraubensäure bestand die Möglichkeit, zu versuchen, sie nach dem Prinzip von CASE (1932) zu bestimmen, indem man Dinitrophenylhydrazin zu Hilfe nimmt, wie man in letzter Zeit häufig verfahren hat. Es muss jedoch bemerkt werden, dass beim Gebrauch der verschiedenen Modifikationen von CASE's Methode oft ausser Acht gelassen wird, dass diese Methode keine unbedingt spezifische Bestimmungsweise der Brenztraubensäure ist. Die Schwankungen, die im Umsatz der Brenztraubensäure beispielsweise bei Anwendung der bekannten Methode von LU (1939) festgestellt worden sind, können offensichtlich zum Teil auf dem Einfluss anderer Ketosäuren beruhen.

Es erwies sich darum als notwendig, zu versuchen, ein Verfahren für Blut zur gleichzeitigen Bestimmung von Brenztraubensäure und α-

Ketoglutarsäure zu entwickeln. Die Versuche, die Brenztraubensäure und die α-Ketoglutarsäure mit Hilfe spezieller Farbreaktionen auf kolorimetrischem Wege zu bestimmen, führten besonders in Bezug auf die α-Ketoglutarsäure zu keinen brauchbaren Ergebnissen. Dagegen schienen bessere Möglichkeiten vorhanden zu sein, dass verschiedene Verhalten der Dinitrophenylhydrazinverbindungen der Brenztraubensäure und der α-Ketoglutarsäure auszunutzen. Abgesehen davon, dass diese Verbindungen in alkalischer Reaktion verschiedene Farbeigenschaften haben, wie ich früher bemerkt habe, (SIMOLA, 1936 a, 1939), besteht auch in ihrer Löslichkeit ein Unterschied. Ausser für qualitative kann man auch für quantitative Zwecke die Tatsache ausnutzen, dass die Dinitrophenylhydrazinverbindung der α-Ketoglutarsäure in alkoholischer Lauge schwer löslich ist. Unter Ausnutzung eben dieser Eigenschaft wurde in einer Anzahl von Fällen versucht, das Auftreten von Brenztraubensäure und α-Ketoglutarsäure im Blut zu ermitteln. Sowohl die Brenztraubensäure als auch die α-Ketoglutarsäure wurden zuerst als Dinitrophenylhydrazinverbindung ausgeschieden, danach wurden die Verbindungen mit Hilfe von alkoholischer Lauge voneinander getrennt, wonach auf Grund der Farbe der Dinitrophenylhydrazinverbindung die Brenztraubensäure und dann in gleicher Weise kolorimetrisch auch die α-Ketoglutarsäure bestimmt wurde. Das Verfahren wird in anderem Zusammenhang genauer beschrieben.

In einigen Fällen musste man sich bei der Bestimmung der α-Ketosäuren nur mit der Bestimmung der bisulfitbindenden Substanzen begnügen, wozu die Modifikation von ELLIOT, BENOY und BAKER (1935) angewandt wurde.

Harn konnte verhältnismässig reichlich erhalten werden, und bei Harnuntersuchung konnte ausser der oben erwähnten Methode auch das von KRUSIUS (1938, 1940) angewandte Verfahren zu Hilfe genommen werden, dessen Prinzip darin besteht, dass die Brenztraubensäure zuerst durch Reduzierung zu Milchsäure, und die α-Ketoglutarsäure durch Oxydierung ihrer Dinitrophenylhydrazinverbindung zu Bernsteinsäure bestimmt wird.

Zur Bestimmung der Citronensäure im Blut wäre die Möglichkeit vorhanden gewesen, die enzymatische Methode von THUNBERG (1929) zu benutzen, welche in anderem Zusammenhang bei der Durchführung von Bestimmungen der Citronensäure im Blut in diesem Laboratorium im Gebrauch war. Es war jedoch gewöhnlich nicht möglich, dass das aus den Versuchstieren erhältliche Blut ausser zur Bestimmung der Brenztraubensäure und der α-Ketoglutarsäure auch noch zur Bestimmung der Citronensäure ausreichte, weshalb man sich bei den Bestimmungen der Citronensäure nur auf den Harn beschränkte. Beim Harn erwies es sich wiederum in der Praxis zweckmässiger, die bekannte Methode von PUCHER, SHERMAN und VICKERY (1936) anzuwenden. — Wegen methodischer Schwierigkeiten war es dagegen nicht möglich das Vorkommen und Verhalten der Isocitronensäure zu untersuchen, obgleich dieser Verbindung als Zwischenprodukt offenbar eine wichtigere Rolle als der Citronensäure zukommt.

Als Versuchstiere wurden Ratten mit einem Anfangsgewicht von 50—150 g verwendet. Die zum Vergleich benutzten Kontrolltiere erhielten als B-Vitaminquelle Hefe. B-Vitaminmangelzustände wurden in folgenden Gruppen angeordnet: Gänzlicher B-Vitaminmangel, B_1-Vitamin allein oder zusammen mit Lactoflavin als Vitaminquelle und autoklavisierte Hefe allein oder zusammen mit B_1 als B-Vitaminquelle. Es wurden auch andere B-Vitaminmangelzustände angeordnet. Die Versuche misslangen jedoch mehr oder weniger, und Resultate werden in diesem Zusammenhang nicht veröffentlicht.

Von B_1-Vitamin und Lactoflavin wurde das etwa Fünf- bis Zehnfache der dem Mindestbedarf einer Ratte entsprechenden Menge verabreicht. Der Hefe wurde vor dem Autoklavisieren etwas Alkali zugesetzt. Was die Anordnung der Avitaminoseversuche im übrigen betrifft, so verweise ich auf die früheren Arbeiten aus diesem Laboratorium (KRUSIUS und SIMOLA, 1937; SIMOLA, 1939).

Das Blut wurde unmittelbar nach Dekapitieren in eine Trichloressigsäurelösung hineinpipettiert.

Der Harn wurde mit Hilfe von Harntrichtern aufgenommen. Während des Sammelns waren die Tiere ohne Nahrung. Um die Einwirkung des Hungers zu eliminieren wurde das Sammeln des Harns jeweils nur auf 12 Stunden ausgedehnt. Auf der angewandten Methodik beruht, dass die Menge der pro Zeiteinheit im Harn ausgeschiedenen Stoffe relativ kleiner war als dann, wenn der Harn in der üblichen Weise im Verlauf von 24 Stunden gesammelt wird, und die Versuchstiere ungehindert Nahrung zu sich nehmen können.

Mit dem Sammeln des Harns wurde begonnen, sobald das Wachstum der Versuchstiere anfing stehenzubleiben, und die Entnahme der Harnproben wurde mit kurzen Pausen so lange fortgesetzt, bis die Mangelerscheinungen weit vorgeschritten waren. Auf Grund der Werte der Harnproben wurde die durchschnittliche Ausscheidung der zu untersuchenden Stoffe während des Mangelzustandes berechnet. Die in der Ausscheidung vorkommenden Schwankungen während der verschiedenen Stadien des Mangelzustandes werden im Folgenden nicht näher beschrieben.

In Bezug auf die Ausscheidung der in Frage stehenden Substanzen bestanden schon zwischen den Individuen der gleichen Tiergruppe bedeutende Unterschiede. Der Vergleich zwischen den verschiedenen Gruppen war in vielen Fällen nur auf Grund von aus verhältnismässig zahlreichen Einzelfällen errechneten Mittelwertzahlen möglich. Vom Standpunkt des Vergleichs aus erwies es sich auch als notwendig, das Gewicht der Ratten zu berücksichtigen. Die im Folgenden angeführten Zahlen beziehen sich auf 1,000 g Rattengewicht.

Versuche.

Im Folgenden werden zuerst einige Ergebnisse aus Versuchsreihen wiedergegeben, worin beabsichtigt wurde, bei der Bestimmung von Brenztraubensäure und α-Ketoglutarsäure das oben

erwähnte kolorimetrische Bestimmungsverfahren anzuwenden. Von den Mangeltieren wurde eine Gruppe in gänzlichem B-Vitaminmangel gehalten. Bei den anderen Gruppen wurde als B-Vitaminquelle B_1-Vitamin oder autoklavisierte Hefe verwendet.

Harn.

Die Resultate der Harnuntersuchungen sind in der folgenden Tabelle wiedergegeben.

Tabelle 1.

Ausscheidung in mg auf 1,000 g Rattengewicht in 12 Stunden.

	Brenztraubensäure	α-Ketoglutarsäure	Citronensäure
Gänzlicher B-Vitaminmangel	2.6	13.0	1.3
B_1-Vitamin als B-Vitaminquelle	1.0	1.0	1.3
Autoklavisierte Hefe als B-Vitaminquelle	2.5	57.8	1.5
Kontrolltiere	1.8	2.9	1.5

Wie aus der Tabelle erhellt, scheint die Brenztraubensäuremenge bei gänzlichem Mangel an B-Vitamin und bei Benutzung von autoklavisierter Hefe als B-Vitaminquelle grösser zu sein als bei den Kontrolltieren, wenn die Differenz auch sehr klein ist. Merkwürdigerweise ist bei Tieren, welche B_1-Vitamin als einziges B-Vitamin erhalten haben, die Brenztraubensäuremenge verhältnismässig niedrig.

Übereinstimmend mit früheren halbquantitativen Bestimmungen war dagegen sowohl bei gänzlichem Mangel des B-Komplexes als auch besonders bei Anwendung von autoklavisierter Hefe als B-Vitaminquelle eine sehr starke Zunahme des α-Ketoglutarsäuregehaltes im Harn festzustellen. Im ersteren Fall war die Steigerung ca vierfach, im letzteren zwanzigfach. In einzelnen Fällen konnte im Harn sogar 0.6 % α-Ketoglutarsäure auftreten. Bei Anwendung von B_1-Vitamin als B-Vitaminquelle war die Menge der ausgeschiedenen α-Ketoglutarsäure auffallend gering.

In Bezug auf die Ausscheidung von Citronensäure zeigen die Mittelwerte keine deutlichen Unterschiede. Bei Tieren, die keine deutlichen Mangelsymptome aufwiesen, konnten bisweilen relativ hohe Citronensäurewerte festgestellt werden. Bei hoch entwick-

elten Mangelzuständen wiederum wurden oft recht geringe Mengen Citronensäure ausgeschieden.

Blut.

In der untenstehenden Tabelle sind die Mittelwerte aus einer Anzahl die Menge der bisulfitbindenden Substanzen des Blutes betreffenden Bestimmungen wiedergegeben, welche wegen der Einfachheit der Methodik leicht durchzuführen waren. Die Anzahl der Bestimmungen in jeder Gruppe betrug 6—20.

Tabelle 2.

Bisulfitbindende Substanzen als Brenztraubensäure berechnet in mg %.

Gänzlicher B-Vitaminmangel	6.2
B_1-Vitamin als B-Vitaminquelle	7.9
B_1-Vitamin und Lactoflavin als B-Vitaminquelle	6.9
Autoklavisierte Hefe als B-Vitaminquelle	7.1
Kontrolltiere	4.8

Im Vergleich zu den Werten der Kontrolltiere ist in sämtlichen Mangelgruppen die Menge der bisulfitbindenden Substanzen deutlich gestiegen. In diesem Falle war der Gehalt an bisulfitbindenden Substanzen bei Anwendung des B_1-Vitamins im Mittel viel höher als früher festgestellt worden war. Der Anstieg des Mittelwertes wurde dadurch verursacht, dass von den 12 Werten der Gruppe 4 aussergewöhnlich hoch waren, während die übrigen normal oder nur verhältnismässig wenig grösser als normal waren. — In diesem Zusammenhang sei erwähnt, dass bei B-Vitaminmangeltieren, z. B. bei mit autoklavisierter Hefe gefütterten, der Gehalt an bisulfitbindenden Substanz im Blut mitunter merkwürdig hoch sein kann, wobei er mehr als 20 mg% entspricht. Am Anfang hatte es den Anschein, als ob es sich um irgendeinen auf technischen Umständen beruhenden Versuchsfehler handelte. Auf Grund späterer Beobachtungen scheint sich jedoch zu ergeben, dass diese Erscheinung auf einer Zunahme der Ketoverbindungen beruht.

Was die nach dem neuen Prinzip ermittelten Brenztraubensäure- und α-Ketoglutarsäurewerte im Blut betrifft, so ist die Angabe der Mittelwertzahlen schwierig, was darauf beruht, dass sich die Methodik noch unter Entwicklung befand, und die Grösse der erhaltenen Werte zu verschiedenen Zeiten etwas schwankte. In den meisten Fällen wurde für den Normalwert der Brenz-

traubensäure im Blut von Normaltieren 1.4—2.2 mg% erhalten. Im Vergleich zu den Kontrolltieren konnte bei den Tieren der Mangelgruppen nicht immer ein deutlicher Unterschied angetroffen werden, was zum Teil darauf beruht, dass die Mangelsymptome in den untersuchten Fällen noch nicht weit genug entwickelt waren. Die deutlichste und gleichmässigste Neigung zum Anstieg war in der Gruppe der mit autoklavisierter Hefe gefütterten Ratten festzustellen, wo 9 von 10 Brenztraubensäurebestimmungen 2 mg% überstiegen, wobei der Höchstwert 3.4 mg% betrug. Bei Ausschluss des ganzen B-Komplexes war der höchste Brenztraubensäurewert 3.8 mg%, andererseits ergaben die Analysen bei totaler B-Vitaminkarenz auch relativ niedrige Werte.

Hinsichtlich des Gehalts an α-Ketoglutarsäure stellte es sich heraus, dass diese Säure bei Normaltieren im Blut so wenig vorhanden war — unter 1 mg% — dass ihre genaue Bestimmung aus kleinen Blutmengen sehr schwierig war. In einigen Fällen waren die Werte bei Normaltieren deutlich höher, aber die Frage, ob dies auf einem methodischen Fehler oder tatsächlich auf einem höheren α-Ketoglutarsäuregehalt beruht, blieb ungeklärt. Was die Gruppen der Mangeltiere betrifft, so sei erwähnt, dass der höchste im Verlauf dieser Untersuchung festgestellte α-Ketoglutarsäurewert, 5.2 mg%, in der Gruppe der mit autoklavisierter Hefe ernährten Ratten festgestellt wurde. In den meisten Fällen war die Steigerung so gering, dass ein wirklich überzeugender Unterschied im Vergleich zu den Normaltieren nicht vorhanden war.

Hiernach mögen Resultate aus einer anderen Versuchsreihe wiedergegeben werden, wo man sich in Bezug auf Harn auf die Bestimmung der α-Ketoglutarsäure und der Citronensäure, und in Bezug auf Blut die der bisulfitbindenden Substanzen beschränkte. Bei der Bestimmung der α-Ketoglutarsäure im Harn wurde hierbei KRUSIUS' Methodik zu Hilfe genommen. In Bezug auf die Deckung des B-Vitaminbedarfes waren folgende Gruppen Gegenstand der Untersuchung: Totaler B-Vitaminmangel, als B-Vitaminquelle autoklavisierte Hefe und als B-Vitaminquelle autoklavisierte Hefe und B_1-Vitamin.

Harn.

Die Mittelwerte der Harnbestimmungen sind in folgender Tabelle zusammengestellt.

Tabelle 3.

Ausscheidung in mg auf 1,000 g Rattengewicht in 12 Stunden.

	α-Ketoglutarsäure	Citronensäure
Gänzlicher B-Vitaminmangel	—	1.9
Autoklavisierte Hefe als B-Vitaminquelle	14.7	2.1
Autoklavisierte Hefe und B_1-Vitamin als Vitaminquelle	3.6	3.2
Kontrolltiere	1.4	2.0

Wie aus der Tabelle hervorgeht, ist in der mit autoklavisierter Hefe gefütterten Rattengruppe, wo B_1-Vitaminmangel am ehesten in Frage kam, im α-Ketoglutarsäuregehalt wiederum eine Steigerung festzustellen, wenn auch diesmal nicht so stark wie in der früher erwähnten Versuchsreihe, wo die α-Ketoglutarsäure mit einem anderen Verfahren bestimmt wurde. Bei Anwendung von B_1-Vitamin als Komplettierung der autoklavisierten Hefe sank die Ausscheidung von α-Ketoglutarsäure, jedoch nicht gänzlich auf das Niveau der Normalwerte.

In den Mittelwerten der Citronensäure sind keine grösseren Underschiede wahrzunehmen. Aus irgendeinem Grunde sind in diesen Gruppen die Mengen der im Harn ausgeschiedenen Citronensäure durchweg etwas höher als in den oben besprochenen Reihen.

Blut.

Die Resultate der Bestimmung von bisulfitbindenden Substanzen bei dieser Versuchsreihe sind in der folgenden Tabelle widergegeben. Die Anzahl der Bestimmungen bei den angeführten zwei Tiergruppen betrug je 10.

Tabelle 4.

Bisulfitbindende Substanzen als Brenztraubensäure berechnet in mg %.

Autoklavisierte Hefe als B-Vitaminquelle	9.5
Autoklavisierte Hefe und B_1-Vitamin als B-Quelle	3.9

Wie bei der früher aufgeführten Versuchsreihe war der Gehalt an bisulfitbindenden Substanzen bei Fütterung mit autoklavisierter Hefe deutlich erhöht. Durch Komplettierung der autoklavisierten Hefe mit B_1-Vitamin wurde die Menge der bisulfitbindenden Substanzen sehr deutlich gesenkt. Der Mittelwert war sogar etwas niedriger als gewöhnlich bei Normaltieren.

Besprechung der Ergebnisse.

Die oben dargestellten Ergebnisse stehen zum grössten Teil im Einklang mit den früher in diesem Laboratorium gemachten Beobachtungen betreffend der Beziehung zwischen der B-Vitamingruppe und dem α-Ketosäurestoffwechsel.

Bei B-Vitaminmangelzuständen wie bei Ausschluss des ganzen B-Vitaminkomplexes und besonders bei Anwendung von autoklavisierter Hefe als B-Quelle tritt als typische Mangelerscheinung eine erhöhte Ausscheidung der α-Ketoglutarsäure auf. Hauptgrund für diese Erscheinung ist der Mangel an B_1-Vitamin. Im Brenztraubensäuregehalt des Harns dagegen ist jedenfalls keine auffällige Steigerung festzustellen. Dagegen ist es offensichtlich, dass die Brenztraubensäure bei der im Blut auftretenden Anhäufung der Ketosäuren beteiligt sei, die im Zusammenhang mit der gesteigerten Ketosäureausscheidung bei B-Vitaminmangeltieren in Erscheinung tritt.

Die Frage, in welchem Umfang die übrigen Faktoren des B-Vitaminkomplexes Einfluss auf diese im α-Ketosäureumsatz auftretenden Erscheinungen haben, wurde auch in diesen Versuchsreihen nicht endgültig geklärt. Bei Anwendung von B_1-Vitamin als Komplettierung der autoklavisierten Hefe blieb die Menge der bisulfitbindenden Substanzen im Blut relativ niedrig, aber die im Harn ausgeschiedene Menge α-Ketoglutarsäure war bei Bestimmung mit KRUSIUS' Verfahren etwas grösser als gewöhnlich. Es ist möglich, dass dies darauf zurückzuführen ist, dass der autoklavisierten Hefe vor der Autoklavisierung etwas Alkali zugesetzt wurde. Andererseits ist es nicht ganz unmöglich, dass hierauf der Mangel eines bei der Autoklavisierung vernichteten Faktors des B-Vitaminkomplexes Einfluss gehabt habe. Wenn B_1-Vitamin allein als B-Quelle verabreicht wurde, war merkwürdigerweise im Gegenteil der α-Ketoglutarsäuregehalt im Harn verhältnismässig niedrig, die Menge der bisulfitbindenden Substanzen im Blut dagegen gestiegen.

Die Frage, weshalb die Wirkung des B_1-Vitaminmangels im Harn in Form einer erhöhten α-Ketoglutarsäureausscheidung erscheint, war im früheren Stadium schwer zu klären. In die Frage kam später mehr Licht durch eine Menge in diesem Laboratorium durchgeführter Untersuchungen, bei denen der Umsatz der Brenztraubensäure mit Hilfe von in vivo- und in vitro-Versuchen

näher untersucht wurde. Bei diesen Untersuchungen stellte sich u. a. heraus, wie schon früher erwähnt wurde, dass das Natriumsalz der Brenztraubensäure eine Steigerung der Ausscheidung von α-Ketoglutarsäure und Citronensäure verursacht. Im Brenztraubensäuregehalt des Harns tritt dagegen keine grössere Steigerung hervor (SIMOLA, 1937, 1938, a; SIMOLA und KRUSIUS, 1939; KRUSIUS, 1940). In späteren Versuchen, die noch nicht veröffentlicht worden sind, wurde u. a. gleichzeitig nach Verabreichung von Brenztraubensäure die Menge der Brenztraubensäure und der α-Ketoglutarsäure im Blut und im Harn bestimmt und festgestellt, dass die Erhöhung des α-Ketoglutarsäuregehaltes im Blute sehr deutlich auftreten kann, indem gleichzeitig im Harn um ein Vielfaches reichlicher α-Ketoglutarsäure als Brenztraubensäure ausgeschieden wird.

Wenn auch diese Wirkung der Brenztraubensäure auf die Erhöhung der Ausscheidung an α-Ketoglutarsäure keine spezifische Erscheinung für die Brenztraubensäure ist, wie schon früher bemerkt wurde, so gaben diese Resultate doch jedenfalls dazu Anlass, die Möglichkeit in Betracht zu ziehen, dass die Ursache für die Anhäufungen von α-Ketoglutarsäure bei Mangeltieren die durch den Mangel an B_1-Vitamin hervorgerufene Zunahme von Brenztraubensäure in den Geweben sei.

Die Erklärung dieser Erscheinung wird jedoch dadurch erschwert, dass die Frage der Wirkungsweise des B_1-Vitamins in ihren Einzelheiten noch unklar ist. Dass B_1-Vitamin als Aktivator beim Stoffwechsel der Brenztraubensäure benötigt wird, ist sicher. Man könnte sich denken, dass die beobachtete fördernde Wirkung des B_1-Vitamins auf verschiedene Reaktionen im Stoffwechsel der Brenztraubensäure darauf beruht, dass die Richtung der Wirkung des B_1-Vitamins von der Art der damit wirkenden Fermentproteine abhängig ist. Andererseits ist zu bedenken, dass auf die Entstehung der verschiedenen Reaktionsprodukte auch andere Faktoren einwirken können, wie z. B. der Umstand, in welchen Masse in Zellen andere reaktionsfähige Verbindungen, z. B. Oxalessigsäure oder Acetaldehyd, gebildet werden, welche mit der Brenztraubensäure oder irgendeinem daraus entstehenden labilen Zwischenprodukt reagieren können. In welchem Umfange B_1-Vitamin im Stoffwechsel der übrigen α-Ketosäuren benötigt wird, ist vorerst eine sehr unklare Frage. Nach MCGOWAN und PETERS (1937) ist die im Taubengehirn vorkommende Brenztraubensäureoxydase, als deren Aktivator B_1-Vitamin funktio-

niert, ohne Wirkung auf α-Ketoglutarsäure. Gewisse Befunde haben jedoch später daraufhin gedeutet, dass B_1-Vitamin auch Bedeutung im Stoffwechsel der α-Ketoglutarsäure habe. Unter der Voraussetzung, dass das B_1-Vitamin tatsächlich ein im Stoffwechsel der α-Ketoglutarsäure notwendiger Faktor sei, könnte man sich denken, dass die Anhäufung von α-Ketoglutarsäure ihrerseits darauf beruht, dass neben Verhinderung des Brenztraubensäureumsatzes bei B_1-Vitaminmangel auch eine Verlangsamung des weiteren Abbaus der α-Ketoglutarsäure auftritt. Der Umstand, dass im Harn im Vergleich zur Brenztraubensäure ganz besonders reichlich gerade α-Ketoglutarsäure ausgeschieden wird, wird hierdurch jedoch nicht in völlig befriedigender Weise erklärt.

Hinsichtlich einer möglichen wirklichen Vermehrung der α-Ketoglutarsäurebildung liegt der Gedanke nahe, dass die α-Ketoglutarsäure aus Glutaminsäure entstanden sei. Eine solche Möglichkeit wird durch die neuen Befunde über die durch Brenztraubensäure hervorgerufene Umaminierung (BRAUNSTEIN und KRITZMANN, 1937, 1939) gestützt. In diesem Laboratorium wurde auch seinerzeit gefunden, dass der alleinige Zusatz von Brenztraubensäure zu tierischen Geweben in vitro eine Bildung von α-Ketoglutarsäure und Alanin hervorrufen kann (SIMOLA und ALAPEUSO, 1939, 1943). Nach KRITZMANN (1940) soll zwar die Umaminierung bei B_1-Vitaminmangel herabgesetzt sein. Andererseits konnten BARRON, LYMAN, LIPTON und GOLDINGER (1941) keine Wirkung des B_1-Vitamins auf die Umaminierung feststellen. Es wäre auf jeden Fall wichtig gewesen festzustellen, ob neben der Zunahme der α-Ketoglutarsäure eine Vermehrung des Alaningehaltes im Harn und im Blut auftritt. Die besonderen Umstände machten jedoch leider die der Durchführung der Bestimmung des Alanins gewidmeten weiteren Untersuchungen unmöglich.

Was im übrigen die Bildung der α-Ketoglutarsäure betrifft, schien von Anfang an Grund vorzuliegen, die Möglichkeit in Betracht zu ziehen, dass α-Ketoglutarsäure auch von selbst aus Brenztraubensäure entstehen könnte, und es war eben diese Möglichkeit, der etwas später besondere Beachtung zugewandt wurde (vgl. SIMOLA, 1938 a, b; SIMOLA und KRUSIUS, 1939; SIMOLA und ALAPEUSO, 1938, 1939, 1943).

In Anbetracht der Befunde, dass aus der Citronensäure enzymatisch α-Ketoglutarsäure gebildet wird (MARTIUS und KNOOP,

1937), und dass in Gewebebreiversuchen aus Brenztraubensäure und C_4-Dikarbonsäuren eine deutliche Citronensäuresynthese festzustellen ist (SIMOLA und ALAPEUSO, 1938; SIMOLA, HALLMAN und ALAPEUSO, 1939; HALLMAN und SIMOLA, 1939; HALLMAN, 1939, 1940) schien Grund vorhanden, die Möglichkeit einer Entstehung der α-Ketoglutarsäure über die Citronensäure in Betracht zu ziehen.

Dadurch waren aber nicht alle experimentellen Befunde zu erklären, und es erhob sich auch die Frage, ob die Brenztraubensäure auf irgendeinem noch unbekannten Wege in α-Ketoglutarsäure übergeführt werden kann. Auf Grund gewisser experimenteller Befunde erscheint es möglich, dass aus Brenztraubensäure und Oxalessigsäure auf einem anderen Weg als über Citronensäure α-Ketoglutarsäure gebildet werden könnte. — Die Hypothese, dass aus Essigsäure und Brenztraubensäure α-Ketoglutarsäure gebildet werde (KREBS, 1936), war dagegen experimentell nicht zu bestätigen, wenn auch einige orientierende Versuche anfangs in gewissem Masse für diese Möglichkeit zu sprechen schienen. (Eine andere Frage ist, ob möglicherweise eine beim Zerfall der Brenztraubensäure entstehende Vorstufe der Essigsäure mit der Brenztraubensäure reagiert unter Bildung von α-Ketoglutarsäure.)

In diesem Zusammenhang kann erwähnt werden, dass die letzten Befunde darauf hingedeutet haben — vor allem die Versuche von EVANS und SLOTIN (1940) mit radioaktivem Kohlendioxyd — dass ausser bei Mikro-Organismen, wie WOOD und WERKMAN (1938, 1939, 1940) festgestellt haben, auch im Tierorganismus eine Synthese von Oxalessigsäure aus Kohlendioxyd und Brenztraubensäure möglich ist. An und für sich könnte hiermit vielleicht die Erscheinung erklärt werden, dass bei Verabreichung von Alkali im Harn neben Citronensäure erhöhte Mengen α-Ketoglutarsäure ausgeschieden werden, während im Gehalt an Brenztraubensäure, Milchsäure und Acetonkörper keine deutliche Steigerung wahrzunehmen ist. Man könnte sich denken, dass die Erhöhung daher kommt, dass durch die Einwirkung des Alkali der Bicarbonatgehalt im Organismus zunimmt, woraus wiederum eine Zunahme der Bildung von Oxalessigsäure folgt.

Es sei noch erwähnt, dass KREBS und EGGLESTON (1940) der Meinung sind, dass B_1-Vitamin bei der Vereinigung von Kohlendioxyd mit Brenztraubensäure zu Oxalessigsäure als Aktivator wirke. Wenn dies der Fall ist, wäre eigentlich zu erwarten, dass

bei B_1-Vitaminmangel im Organismus weniger als gewöhnlich Oxalessigsäure entstünde, und daraus folgte die Verminderung der Entstehung von Citronsäure und α-Ketoglutarsäure, während die Menge der Brenztraubensäure steigt. Dies steht jedoch im Widerspruch mit der Tatsache, dass bei B_1-Vitaminmangel im Gegenteil eine Anhäufung von α-Ketoglutarsäure auftritt. Aber andererseits kann zur Erklärung natürlich bemerkt werden, dass die Menge der im Organismus gebildeten α-Ketoglutarsäure auch von anderen Faktoren abhängig ist. Es wurde ja schon früher u. a. auf die Bedeutung der Glutaminsäure bei der α-Ketoglutarsäurebildung hingewiesen.

Was besonders den Citronensäurestoffwechsel betrifft, so konnte bei B-Vitaminmangelzuständen gleichzeitig beim Steigen des α-Ketoglutarsäuregehaltes im Harn keine Zunahme der Ausscheidung von Citronensäure wahrgenommen werden. In einigen Fälle schien, wenn die Symptome des Mangelzustandes nicht entwickelt waren, relativ reichlich Citronensäure im Harn ausgeschieden zu werden, in späterem Stadium konnten relativ niedrige Werte angetroffen werden. In der durchschnittlichen Citronensäureausscheidung, welche auf die ganze Untersuchungsperiode berechnet war, traten, wie aus den obigen Ausführungen herforgeht, keine deutlichen Unterschiede auf. Auf jeden Fall fehlt bei Mangel an B_1-Vitamin bei der Zunahme der α-Ketoglutarsäureausscheidung ein derartiger deutlicher Anstieg des Citronensäuregehaltes, was bei normalen Tieren gewöhnlich — z. B. bei Verabreichung von Natriumsalz der Brenztraubensäure — mit der Steigerung der α-Ketoglutarsäureausscheidung verbunden ist. Ob dies darauf beruht, dass die Fähigkeit des Organismus, Citronensäure zu synthetisieren, geschwächt ist, oder auf sonst einem den Citronensäurestoffwechsel beeinflussenden Umstand, ist schwer zu sagen. Die Möglichkeit, dass das B_1-Vitamin ein bei der Citronensäuresynthese notwendiger Faktor wäre, wie schon früher angenommen wurde (HALLMAN und SIMOLA, 1939), scheint jedenfalls nicht auszuschliessen zu sein. Die Frage ist dadurch kompliziert, dass der Citronensäurestoffwechsel offenbar von recht verschiedenen Faktoren abhängig ist. Es ist in Betracht zu ziehen, dass auch bei dem Abbauweg der Citronensäure die B-Vitaminkomponenten wahrscheinlich eine Rolle spielen. Bei in vivo-Versuchen ist u. a. auch die Wirkung der Reaktion zu beachten. Durch die Acidose wird bekanntlich die Citronensäureausscheidung stark herabgesetzt.

Um näheren Aufschluss über den Umsatz der Citronensäure zu erhalten, war die Absicht, Belastungsversuche mit verschiedenen organischen Säuren bei Mangeltieren anzuordnen und die Bildung der Citronensäure bei verschiedenen Geweben B-avitaminotischer Tiere zu untersuchen. Auf Grund vorbereitender in vitro-Versuche schien es, dass auch in Mangelzuständen im Gewebebrei eine Synthese der Citronensäure in vitro aus Brenztraubensäure und C_4-Dikarbonsäure erreicht werden kann. In einigen Fällen schien die Synthetisierung jedoch etwas schwächer als normal zu sein. Die Anzahl der Versuche war jedoch wegen Unterbrechung der Untersuchungen so gering, dass es schwierig ist, aus ihnen weiter gehende Schlüsse zu ziehen.

Da die früher in diesem Laboratorium durchgeführten Versuche gezeigt haben, dass die β-Oxybuttersäure und Acetessigsäure als Muttersubstanzen bei der Citronensäurebildung eine wichtige Rolle spielen können (Simola, 1938 a; Simola und Alapeuso, 1938; Hallman und Simola, 1939; Hallman, 1940) kann es angebracht sein, in diesem Zusammenhang zu erwähnen, dass übereinstimmend mit den früheren Beobachtungen in den beschriebenen ergänzenden Versuchen bei Mangel an B-Vitaminkomponenten keine deutlichen Störungen im Stoffwechsel der Acetonkörper festgestellt werden konnten.

Zum Schluss seien einige in der letzten Zeit anderwärts durchgeführte Untersuchungen erwähnt, welche ähnliche Fragen wie die oben besprochenen behandeln.

Banerji und Harris (1939) stellen als neue Beobachtung dar, dass bei B_1-Vitaminmangel im Harn reichlich bisulfitbindende Stoffe ausgeschieden werden, ohne jedoch sagen zu können, um welche Stoffe es sich handelt. Dieselbe Beobachtung haben auch Shils, Day und McCollum (1940, 1941) gemacht. Nach ihnen ist die Vermehrung bisulfitbindender Substanzen auf eine Steigerung der Brenztraubensäureausscheidung zurückzuführen. Über eine Vermehrung des Brenztraubensäuregehaltes bei B_1-Vitaminmangel im Harn berichten auch Harper und Deuel jr. (1941), die aber hinsichtlich der Vermehrung bisulfitbindender Substanzen auch die Rolle verschiedener anderer Harnbestandteile — wie von Allantoin — in Betracht ziehen.

Meine früheren Befunde über die Beziehungen des B_1-Vitamins zu dem Stoffwechsel von α-Ketosäuren sind den Autoren entgangen.

Die starke Erhöhung der Ausscheidung der α-Ketoglutarsäure

bei B_1-Vitaminmangel genügt zwar schon zu einem bedeutenden Teil um zu erklären, warum die Menge der bisulfitbindenden Stoffe im Harn gestiegen ist. Ich habe selbst vor etwa zehn Jahren bei den allerersten Harnuntersuchungen mit B-avitaminotischen Ratten auch die Bisulfitbindungsmethode geprüft, ging aber wegen der schwachen Spezifität des Verfahrens zu anderen Methoden bei Bestimmung von α-Ketosäuren über.

Die angegebenen erhöhten Brenztraubensäurewerte dürften zum grossen Teil auf eine Steigerung des α-Ketoglutarsäuregehaltes zurückzuführen sein. Bestimmung der Brenztraubensäure mittels der Methode von Lu, die man bei den letztgenannten Arbeiten benutzt hat, ist für die Brenztraubensäure, wie ich oben erwähnt habe, kein völlig zuverlässiges Verfahren. Die Farbe der Dinitrophenylhydrazinverbindung der α-Ketoglutarsäure ist im Vergleich mit der Dinitrophenylhydrazinverbindung der Brenztraubensäure in alkalischer Lösung zwar so schwach, dass bei Bestimmung der Brenztraubensäure in üblicher Weise mit Hilfe von Dinitrophenylhydrazin die Gegenwart von relativ geringen Mengen α-Ketoglutarsäure nicht wesentlich stört. Handelt es sich aber um eine so starke Erhöhung des Gehaltes an α-Ketoglutarsäure wie bei B_1-Vitaminmangel im Harn, ist es nicht möglich mittels dieser Methode, etwas Sicheres über den Brenztraubensäuregehalt zu schliessen. Auf jeden Fall ist es begründet, bei Untersuchungen über das B_1-Vitamin und den Stoffwechsel der Brenztraubensäure dem Auftreten und der Bildung von α-Ketoglutarsäure mehr Aufmerksamkeit zuzuwenden als früher.

In Bezug auf die Citronensäure liegt schon aus früherer Zeit die Beobachtung von Krebs (1938) vor, dass bei B_1-Vitaminmangel der Citronensäuregehalt des Harns beträchtlich steige. Später kamen Sober, Lipton und Elvehjem (1940) dagegen zu dem Ergebnis, dass der B_1-Vitaminmangel vermindernd auf die Citronensäureausscheidung der Ratten wirkt. Die Erscheinung trat deutlich besonders im Polyneuritis-Stadium auf. Vorher ging in den Citronensäurewerten eine Steigerung vor sich. Etwas später haben Smith und Meyer (1941) bei B_1-Vitaminmangel ebenfalls eine deutliche Verminderung der Citronensäureausscheidung festgestellt, sie sind jedoch — im Gegensatz zu Sober, Lipton und Elvehjem — der Ansicht, dass dies nicht darauf beruht, dass das B_1-Vitamin in enger Beziehung zum Citronensäurestoffwechsel steht, sondern darauf, dass die Ratten bei B_1-

Vitaminmangel weniger Nahrung zu sich nehmen. Sie hatten ihrerseits jedoch keine Erklärung vorzubringen für die von SOBER, LIPTON und ELVEHJEM gemachte interessante Wahrnehmung, dass im B_1-Vitaminmangel bei Belastung mit bernsteinsäurem Natrium keine so kräftige Steigerung des Citronensäuregehaltes im Harn wie bei den mit B_1-Vitamin gefütterten Tieren eintritt, welcher Umstand seinerseits die Auffassung stützt, dass das B_1-Vitamin eine direkte Wirkung auf den Citronensäurestoffwechsel habe. — Es hat den Anschein, dass gerade durch die Durchführung verschiedener Belastungsversuche bessere Möglichkeiten beständen, bei in vitro-Versuchen die Beziehung des B_1-Vitamins zum Citronensäurestoffwechsel zu untersuchen, als durch sonstige Verfolgung der täglichen Citronensäureausscheidung im Harn, hinsichtlich welcher, wie oben bemerkt wurde, verschiedene Faktoren leicht Schwankungen nach der einen oder anderen Seite hervorrufen können. Es wäre auch wichtig bei den Citronensäureuntersuchungen dem Vorkommen der Isocitronensäure im Tierorganismus Aufmerksamkeit zu widmen.

Zusammenfassung.

Im Anschluss an frühere Untersuchungen des Verfassers werden komplettierende Versuche dargestellt, worin unter Anwendung von quantitativen Methoden der Umsatz von Brenztraubensäure, α-Ketoglutarsäure und Citronensäure bei B-avitaminotischen Ratten untersucht wurde. Bei der gleichzeitigen Bestimmung von Brenztraubensäure und α-Ketoglutarsäure im Harn und Blut wurde ein neues Prinzip zu Hilfe genommen.

Die Resultate gingen in Bezug auf den Umsatz der α-Ketosäuren in gleicher Richtung wie früher. Von den Faktoren des B-Vitaminkomplexes verursacht vor allem der Mangel an B_1-Vitamin eine starke Zunahme des α-Ketosäuregehaltes. Hierbei kommt am ehesten eine Steigerung der Ausscheidung von α-Ketoglutarsäure in Frage. In der Ausscheidung von Brenztraubensäure konnte keine grössere Steigerung festgestellt werden.

Gleichzeitig tritt im Blut eine Zunahme der Carbonylverbindungen auf. Dies ist zum Teil auf die Zunahme der Brenztraubensäuremenge zurückzuführen. Zum Teil kann auch das Auftreten von α-Ketoglutarsäure und möglicherweise irgendwelcher unbekannten Ketoverbindungen der Grund hierfür sein.

Eine deutliche Erhöhung des Gehaltes an Citronensäure im Harn, welche bei Normaltieren häufig mit der experimentell — z. B. durch Verabreichung von Natriumsalz der Brenztraubensäure — hervorgerufenen Steigerung der Ausscheidung von α-Ketoglutarsäure verbunden ist, war bei B-Vitaminmangelzuständen nicht nachzuweisen. Vielmehr konnten bei Endstadien der Avitaminosen mitunter relativ niedrige Citronensäurewerte festgestellt werden.

Literatur.

Banerji, G. G. und L. J. Harris, Biochem. J. 1939, *33*, 1346.
Barron, E. S. G., C. M. Lyman, M. A. Lipton und J. M. Goldinger, J. biol. Chem. 1941, *141*, 957. Ref. Chem. Z. 1943, I, 2213.
Braunstein, A. E. und M. G. Kritzmann, Enzymologia 1937, *2*, 129.
—, —, Ebenda 1939, *7*, 25.
Case, E. M., Biochem. J. 1932, *26*, 753.
Elliot, K. A. C., M. P. Benoy und Z. Baker, Ebenda 1935, *29*, 1938.
Evans, E. A. und L. Slotin, J. biol. Chem. 1940, *136*, 301.
Hallman, N., Suomen Kemistil. B 1939, *12*, 11.
—, Acta physiol. scand. 1940, *2*, Suppl. IV.
—, und P. E. Simola, Suomen Kemistil. B 1938, *11*, 21.
—, —, Science 1939, *90*, 591.
H. H. Harper und H. J. Deuel jr., J. biol. Chem. 1941, *137*, 233.
Knoop, F. und C. Martius, Hoppe-Seyl. Z. 1936, *242*, I.
Krebs, H. A., Nature 1936, *138*, 288.
—, Chem. and. Ind. 1938, *57*, 213.
—, und L. A. Eggleston, Biochem. J. 1940, *34*, 1983.
Kritzmann, M. G., Biochemia 1940, *5*, 281. Ref. Chem. Z. 1943 I, 1488.
Krusius, F.-E., Suomen Kemistil. B. 1938, *11*, 10.
—, Acta physiol. scand. 1940, *2*, Suppl. III.
—, und P. E. Simola, Biochem. Z. 1937, *290*, 428.
—, —, Suomen Kemistil. B 1938, *11*, 24.
Lu, G D., Biochem. J. 1939, *33*, 249.
Martius, C. und F. Knoop, Hoppe-Seyl. Z. 1937, *246*, I.
McGowan, G. K. und R. A. Peters, Biochem. J. 1937, *31*, 1637.
Pucher, G. W., C. C. Sherman und H. B. Vickery, J. biol. Chem. 1936, *113*, 235.
Shils, M. E., H. G. Day und E. V. McCollum, Science 1940, *91*, 341.
—, —, —, J. biol. Chem. 1941, *139*, 145.
Simola, P. E., Biochem. Z. 1932, *254*, 229.
—, Suomen Kemistil. B. 1936 a, *10*, 12.
—, Ebenda 1936 b, *10*, 18.
—, Kongressbericht d. nord. Naturforscher Kongr. in Helsinki 1936 c, 559.
—, Suomen Kemistil. B 1937, *11*, 19.
—, Skand. Arch. Physiol. 1938 a, *80*, 375.

SIMOLA, P. E., Acta med. scand. 1938 b, Suppl. XC, 300.
—, und H. ALAPEUSO, Suomen Kemistil. B 1938, *11*, 17.
—, —, Ebenda 1939, *12*, 9.
—, —, Hoppe-Seyl. Z. 1943, *278*, 57.
—, N. HALLMAN und H. ALAPEUSO, Suomen Kemistil. B 1939, *12*, 10.
—, und T. KOSUNEN, Ebenda, 1938, *11*, 38.
—, und F.-E. KRUSIUS, Ebenda 1938, *11*, 9.
—, —, Hoppe-Seyl. Z. 1939, *261*, 209.
SMITH, A. H. und C. E. MEYER, Ebenda, 1941, *139*, 227.
SOBER, H. A., M. A. LIPTON und C. H. ELVEHJEM, J. biol. Chem. 1940, *134*, 605.
THUNBERG, T., Biochem. Z. 1929, *206*, 109.
WOOD, H. G. und G. WERKMAN, Biochem. J. 1936, *30*, 48.
—, —, Ebenda 1938, *32*, 1262.
—, —, Ebenda, 1940, *34*, 7.

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Fish constitutes an important part of human food consumption. Therefore and in view of the fact that the nutritive value of most of the fish species in Sweden is not yet known, the biological value of the fish protein must be considered as an important problem. This value is closely connected with the amounts of indispensable amino acids present in the protein. Recently this problem has been actualized by the demonstration of EVANS et al. 1940, that the incorporation of fish in the ration of foxes will be followed by a serious disease, the Chastek paralysis. In this paper the amino acids obtained by hydrolyzing the muscle substance of the cod (Gadus calarias), the roach (Rutilus rutilus) and the sprat (Clupea sprattus) were determined.

In preparing the material for hydrolysis the fresh fishes were freed from the skin, viscera, bones and the major pieces of connective tissues. The muscle substance was then boiled for 20 minutes, reduced to a pulp by means of a meat chopper and extracted three times with two times its volume of absolute alcohol and ether until it was freed from the major part of fat. The product was air-dried and powdered by treatment in a rolling-mill for 24 hours. After this procedure it was extracted in a Soxhlet apparatus.

Results and Discussion.

The nitrogen distribution was determined by the electrodialyzing technique as modified by THEORELL (1943). The values given in Table 1 are typical of the different fractions.

Table 1.

The nitrogen distribution of fish muscle. All values in per cent of total nitrogen.

Fraction	The sprat	The roach	The cod
Humin-N	1.6	1.1	1.5
Amide-N	11.0	10.4	8.0
Anodic-N	12.6	11.4	12.4
Neutral-N	45.5	48.0	45.0
Cathodic-N	25.0	22.4	27.8

The results are in good agreement with those obtained by ROSEDALE (1929) with some other fishes. Table 2 shows the results of the amino acid determinations as well as some previously published analysis of some other fishes which are of interest in this connection.

Table 2 demonstrates the advance in quantitative amino acid determinations made during the last years. If the values of alanine not determined in the present investigation are considered to amount to about 5 per cent, (ABDERHALDEN, 1936), 95 per cent yields of amino acids can now be obtained. This means a long stage further in the attempt to elucidate the protein structure. There is but an insignificant difference between the data obtained in the present investigation and the values of a recent investigation of the amino acid composition of the whole fish protein (ÅGREN, 1943). The same species of fish as in the present investigation were investigated.

Certain conclusions may be drawn concerning the nutritional quality of the muscle proteins occuring in the fish. All the amino acids which ROSE (1939) considered to be indispensable owing to their property of stimulating growth are present. It must be emphasized that most of the data available as to the question what amino acids are indispensable were determined on relatively few species and chiefly on the rat as experimental animal. The

Table 2.

The amino acid composition of fish muscle.

The values are expressed as percentage of the moisture and ash free protein.

Amino acid	The sprat Present author	The roach Present author	The cod			The halibut Osborne
			Okuda et. al.	Abderhalden	Present author	
Arginine . . .	7.20	7.30	6.68	6.8	9.0	6.34
Histidine	1.70	1.45	2.29	4.8	1.6	2.55
Lysine	6.0	4.08	8.35	8.0	4.8	7.45
Hydroxylysine .	1.40	1.32	—	—	1.15	—
Tyrosine	3.60	4.60	2.46	2.0	4.75	2.39
Tryptophane . .	0.85	0.68	+	2.1	0.67	2.89
Proline	5.0	7.1	1.68	2.8	5.1	3.17
Hydroxyproline .	0	0	—	0.9	0	—
Threonine . . .	0.58	0.58	—	—	0.57	—
Serine	2.45	4.45	0.51	1.8	3.50	?
Glycine	1.81	2.70	trace	present	1.0	?
Alanine	—	—	3.53	5.7	—	?
Phenylalanine . .	14.0	14.8	2.31	1.8	14.4	3.04
Cystin + cysteine	1.6	1.6	—	0.6	1.8	—
Methionine . . .	2.3	2.2	—	0.3	2.2	—
Leucine	15.5	18.0	2.46	7.5	16.3	—
Isoleucine . . .	—	—	—	1.5	—	—
Valine	0.55	0.64	3.88	3.7	0.66	0.79
Hydroxyglutamic acid	0	0	—	—	0	—
Aspartic acid . .	24.4	20.4	0.6	0.6	22.3	2.73
Glutamic acid .			5.24	7.5		10.1
Ammonia . . .	1.95	1.80	0.75	—	1.50	1.33
	90.9	93.7	40.7	58.5	91.2	50.2

determination of the kinds and amounts of pure amino acids needed by the human being has not yet been more thoroughly investigated. Most likely all higher organisms require the same kinds and amounts of essential amino acids, an assumption which by the way has been advanced by many workers but no definite evidence in support of this generalization has yet been established.

In this connection a comparison with the amino acid composition of the muscle protein of man may be of certain interest. In Table 3 the analysis of human muscle protein from an amputaded leg are listed (SHARPENEK, et al. 1934).

Table 3.

The amino acid composition of human muscle protein.

The values are expressed as percentage of the muscle protein.

Glycine + alanine	4.92	Phenylalanine	5.25
Valine	6.44	Tyrosine	4.11
Leucine	8.88	Tryptophane	2.3
Arginine	8.81	Cystine	2.27
Histidine	2.39	Proline	4.16
Lysine	6.57	Ammonia	1.24
	38.01		59.33

On comparing Tables 2 and 3 it will become evident that the fish protein contains apart from valine all the amino acids recognized to be present in human muscle protein in amounts which should suffice to prevent undue loss of human muscle substance, if fish were the sole resource of protein. The amount of valine present in the human muscle protein is unusually high. The rabbit myosin contains three per cent of valine (SHARP, 1939). Casein with 7.9 per cent yields of valine seems to be the only protein reported to contain a higher concentration (SCHMIDT, 1938). ROSE (1938) reported that animals deprived of valine exhibit unusual symptoms including sensitiveness to touch and lack of musclular coordination. Possibly it was not only a coincidence that, according to the data, foxes fed on fish during long periods, also exhibited these symptoms (EVANS et al. 1940). BURROUGHS and collaborators (1940) also reported that only five amino acids are essential to the fullgrown rat. These amino acids are threonine, isoleucine, trytophane, phenylalanine and valine.

A fractionation of the muscle proteins according to the principles of WEBER (1933) demonstrates that the fish muscle also consists of at least three distinct fractions corresponding to the globulin, myosin, the albumin, myogen and to a globulin X. As in the rabbit-muscle the main fraction of the muscle proteins of fish consists of myosin (ÅGREN, unpublished experiments). The amino acid composition of this protein dominates the data of analysis obtained in the present investigation. Table 2 shows that the dicarboxylic acids together with the basic and hydroxy-

amino acids owing to the fact that they constitute nearly 50 per cent of the total protein provide a large number of polar side-chains. The presence of these chains together with a large number of long non-polar side-chains and a small number of rigid disulphide links would constitute a relatively accessible system of polypeptide chains with many center for the free coordination of water molecules. Furthermore, if one attempted to depict the process of muscular contraction as being due to the action of certain products of metabolism on specific groups in the peptide chains, it is clear that an open structure, such as the one which can be postulated from the chemical data, will favor the rapid diffusion of these substances and their secondary products into and away from the sites of action.

Experimental.

Moisture. A sample was dried to constant weight in vacuum over P_2O_5 at 100° C.

Ash. The residue from the moisture determination was ashed over a Bunsen flame and than kept in a furnace at 600° for 2 hours.

Total Nitrogen. By micro-Kjeldahl analysis.

The Nitrogen Distribution and Amide Nitrogen were determined according to THEORELL (1943) on samples of 150 mg.

Humin Nitrogen. The hydrolysates prepared for the determination of the basic amino acids were filtered through a paper and the residue carefully washed with distilled water and analyzed by the Kjeldahl procedure.

As the amount of material for amino acid analysis was of limited size no attempt was made to use the general solubility method devised by BERGMANN and STERN (1939). The attention was focused only on colorimetric and small scale isolation methods. All the colorimetric determinations were carried out by means of a Zeiss Pulfrich Photometer. The instrument was used in such a manner that the drum-readings obtained by comparing the coloured solutions with appropriate blanks were referred to calibration curves constructed from values obtained with standard solutions of the pure amino acid being estimated. Unless otherwise stated, the results obtained are the mean of two or more determinations and refer to the moisture and ash-free material.

Determination of the Basic Amino Acids.

This determination was made according to the procedure of THEORELL (1943). Samples of 100—150 mg. of hydrolyzed protein were electrodialyzed for about 12 hours. Ammonia was distilled off from the cathode fraction and the remaining solution was re-electrodialyzed. The new cathode fraction was then analyzed for histidine, according to JORPES (1932) and for arginine according to THOMAS, INGALLS and LÜCK (1939), Lysine was obtained as the difference: total base-N —

(arginine- + histidine- + hydroxylysine-N). In using Jorpes method based on diazotation a calibration curve was constructed which showed that an extinction coefficient = 2.35 was adequate under the present conditions. In carrying out the arginine determinations it was found necessary to make use of the correction introduced by KASSEL and BRAND (1942). A typical example of an arginine analysis is given in Table 4.

Table 4.

The determination of arginine in the muscle protein of roach.

ml of cathode fraction to analysis	0.15	0.30	0.40	0.50
Arginine found in mg . . .	0.018	0.034	0.045	0.055
Arginine in mg/ml	0.120	0.113	0.112	0.110
Arginine in per cent	7.15	6.75	6.65	6.55

The corrected value according to KASSEL and BRAND = 7.40 %.

Hydroxylysine. The amino acid was determined by applying the periodic acid method of VAN SLYKE (1940) to the cathode fractions obtained by electrodialysis as described above. This simple method could be used since no other hydroxyamino acid would pass into the cathode compartment of the electrodialysing apparatus. In the determinations three cathode fractions were used together, of which each had been obtained from 100 mg of hydrolyzed protein. The hydroxylysine values obtained agreed fairly well as will be demonstrated by the following example: Cathode fractions obtained by electrodialysis and re-electrodialysis of 300 mg of hydrolyzed protein from cod muscle as described above were concentrated in vacuum to a volume of 10 ml. 5 ml were added to each of the two first tubes in the apparatus described by VAN SLYKE and CULLEN (1916). The titration values obtained in three experiments are given in Table 5.

Table 5.

The determination of hydroxylysine in the cod.

The ammonia set free by treating the amino acid with HIO_4 is titrated with n/100 HCl.

Sample	Periodic acid tube ml n/100 HCl	Control tube ml n/100 HCl	Mg hydroxy-lysine	Hydroxylysine in percentage of ash and moisture free protein
1	49.15	50.0	1.39	1.10
2	49.10	50.0	1.46	1.15
3	49.20	50.0	1.30	1.03

precipitated by means of ammonium rhodanilate according to the method of BERGMANN and NIEMANN (1938). Ammonium rhodanilate was prepared according to BERGMANN (1935). The possibility of precipitating proline on a small scale, as described by DEVINE (1941), was investigated. The following method was used: 6 g of protein were refluxed with 100 ml 20 % HCl for 24 hours. After removing the humin by centrifugation and washing, the total solution was concentrated in vacuum three times to remove the hydrochloric acid. The basic amino acids were precipitated with phosphotungstic acid according to the directions given by VAN SLYKE (1942). It was calculated that about 60 ml 10 % phosphotungstic acid would precipitate the basic amino acids in the proline hydrolysates. After having been kept in the refrigerator over night the phosphotungstic acid precipitation was removed by centrifugation, and filtrate and washings were concentrated to a gum and diluted to 50 ml. Varying amounts of ammonium rhodanilate solved in 4.8 ml of methyl alcohol were added to 6 aliquots (volume = 8 ml) of the amino acid solution. After 2 hours on ice the mixtures were filtered on sintered glass crucibles and the residue washed three times with ice-cold water and dried in a desiccator. The weights and nitrogen contents of the precipitates were determined. By plotting the amount of nitrogen present in the proline rhodanilate against the weight of the ammonium rhodanilate used in precipitation, an inflection point on the curve could be observed. This point presumably indicated the most favourable conditions for the precipitation of proline rhodanilate. In Table 6 a typical analysis is demonstrated.

Table 6.

The determination of proline in the roach.

Gm ammoninmsalt used in precipitation	Nitrogen content of proline precipitate in per cent	Weight of proline salt in gm	Nitrogen content in proline salt in mg
0.1	16.6	0.043	7.15
0.2	16.6	0.132	22.2
0.3	16.4	0.194	32.2
0.4	16.5	0.256	42.5
0.5	17.3	0.333	58.0
0.6	17.4	0.442	77.0

The series of values gives an inflection point corresponding to 0.45 g of ammonium salt, which would precipitate 290 mg of proline salt. Correcting for the amount of soluble proline salt, this yields 0.06 g of proline per 0.84 g of protein = 7.1 % of proline.

Hydroxyproline. No small scale gravimetric method has yet been developed for the determination of hydroxyproline. The colorimetric method of WALDSCHMIDT-LEITZ and AKABORI (1934) is reported to give low values. The principle of oxidizing the hydroxyproline and

given by BLOCK (1938). The values were corrected for unprecipitated glycine according to BERGMANN (1938).

Phenylalanine. In the first series of determinations the KAPELLER-ADLER colorimetric method as modified by BLOCK (1938) was used. It was found necessary to dilute the amino acid hydrolysate to 30 ml. 3 ml were used for each determination. BLOCK when modifying the method considered the removal of basic amino acids negligible if the colour was measured with the aid of a special filter. The high values obtained with this modified method necessitated control series which were carried out by the original method on hydrolysates precipitated with phosphotungstic acid. The procedure outlined by DEVINE (1941) was followed. Typical values obtained with the two methods are listed in Table 7.

Table 7.

The determination of phelylalanine in fish protein.

Values in percentage of ash and moisture free protein.

	Block's method	Devine's method
Cod	18.0	14.4
Roach	17.9	14.8
Sprat	18.5	14.0

The values obtained with DEVINE's method were chosen, as they express more specifically the true phenylalanine contents.

Cystine and Methionine. By the method of BAERNSTEIN (1936) methionine can be most accurately determined on a small scale, but it requires a specially designed apparatus. The method is also reported to give unsatisfactory yields when applied to certain proteins. The gravimetric method of BEACH and TEAGUE (1942) was therefore used. This method enables the determination of both cystine + cysteine. and methionine. Cystine is reduced to cysteine. Methionine is demethylated and the homocysteine-thiolactone which is formed, does not directely form a mercaptide with Cu_2O as cysteine will do. If the solution of homocysteine and cysteine is treated with alkali, both compounds are precipitated as mercaptides. The difference between the two sets of values corresponds to methionine. The method was first tested with mixtures of pure cysteine and methionine. The cysteine values obtained were somewhat to low, as is demonstrated in Table 8.

The low values are due to the tendency of cysteine to be partly oxidized to cystine during the removal of the last amounts of hydroiodic acid which is used in hydrolyzing the protein. The error could be eliminated by using nitrogen atmosphere during the vacuum distillation of the hydroiodic acid. A few other modifications of the method were utilized. The methionine values were substantially improved by treating the cysteine-homocysteine solutions with Cu_2O during 45

seconds. The mercaptides were solved with the aid of 2—3 ml of concentrated HNO_3 and 1 ml of DENIS' copper reagent. The solution was evaporated on wather-bath before ashing. The methionine values were corrected by the empirical factor of BEACH and TEAGUE.

Table 8.

Determination of cysteine-methionine mixtures.

Sample	Calculated in mg	Found in mg
Cysteine	7.8	7.0
Cysteine	7.8	7.1
Methionine	4.0	3.9
Methionine	4.0	4.0

Leucine. The method of BARNETT (1933) was followed. In the determination 100 g of protein were refluxed with 280 ml of concentrated hydrochloric acid for 18 hours. As tyrosine did not show any tendency to crystallize out separatedly, it was necessary to purify the crude products according to COX, KING and BERG (1929).

Valine. This amino acid was determined according to the colorimetric method outlined by WRETLIND (1942). The first step in the reaction is an oxidation of valine to the corresponding aldehyde, isobutyric aldehyde, which subsequently after steamdistillation in a specially designed apparatus is allowed to react with salicylic aldehyde. The reaction product is red coloured and can be photometrically determined. WRETLIND gives the following empirical relation:

$$\text{valine} = \frac{a}{3} \times 108 \times E^{1}_{50}$$

were a = ml solution obtained by steam-distillation and E = extinction coefficient obtained with the Zeiss-Photometer using filter S 50 and 1 cm cups. In a series of determination with 60—500 γ of valine it was found that the obtained values better satisfied the following relation:

$$\text{valine} = \frac{a}{3} \times 210 \times E^{1}_{50}$$

This formula was used in the present investigation. Since WRETLIND stated that contamination of leucine in the determination would increase the colour, all valine determinations were carried out on solutions freed from leucine by the BARNETT's procedure (1933). Since the valine values obtained on the leucine-free hydrolysates were very low, it was suspected that valine might have precipitated together with leucine. The leucine fractions were investigated, and it was found that less than 0.03 % of valine had been lost in the leucine precipitates.

Summary.

The amino acid composition of the muscle protein from some Swedish fishes, commonly used for food consumption has been investigated. The amino acids determined correspond to more than 90 per cent by weight of the protein. By incorporation of the alanine values obtained by other authors, more than 95 per cent of the amino acid content has been recognized. The nutritional quality of the fish protein must be classified as rather high since all the indespensable amino acids — possibly with the exception of valine — are present in amounts which corresponds to those reported to be present in rabbit- and human muscle protein.

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References.

ABDERHALDEN, E., E. BAERTICH and W. ZIESECHE, Hoppe Seyl. Z., 1936, *240*, 152.
BAERNSTEIN, H. D. J. Biol. Chem., 1936, *110:* 25.
BARNETT, H. M., Ibidem, 1933, *100:* 543.
BEACH, E. F., and D. M. TEAGUE, Ibidem, 1942, *142:* 277.
BERGMANN, M., Ibidem, 1935, *110:* 471.
—, Ibidem, 1938, *122:* 569.
BERGMANN, M., and C. NIEMANN, Ibidem, 1938, *122:* 577.
BERGMANN, M., and G. STEIN, Ibidem, 1939, *128:* 217.
BURROUGHS, E. W., H. S. BURROUGHS and H. H. MITCHELL, J. Nutrit., 1940, *19:* 363.
COX, G. J., KING, H., and C. P. BERG, J. Biol. Chem., 1929, *81:* 755.
DEVINE, J., Biochem. J., 1941, *35:* 433.
EVANS, C. A., R. G. GREEN and W. E. CARLSON, J. Nutrit., 1941, *21:* 243.
FOLIN, O., and A. D. MARENZI, J. Biol. Chem., 1929, *83:* 103.
GUEST, G. H., Canad. J. Res., 1939, *17 (B):* 143.
JORPES, E., Biochem. J., 1932, *26:* 1507.
KASSEL, B., and E. BRAND, J. Biol. Chem., 1942, *145:* 359.
LAPRAIK, W., J. prakt. Chem., 1893, *47:* 305.
LOWNDES, T. J., MACARA, R., and R. H. A. PLIMMER, Biochem. J., 1941, *35:* 315.
LUGG, J. W. H., Ibidem, 1933, *27:* 668.

Mc FARLANE, W. D., and G. H. GUEST, Canad. J. Res., 1939, *17 (B):* 139.
NICOLET, B. H., and L. A. SHINN, J. Biol. Chem, 1941, *138:* 91.
—, —, Ibidem, 1941, *139:* 687.
—, —, Ibidem, 1942, *142:* 141.
OSBORNE, T. B., and T. W. HEYL, Amer. J. Physiol., 1908—09, *23:* 81.
OKUDA, Y., and O. KIYOSHI, J. Col. Agr. Imp. Univ. Tokyo, 1916, *5:* 365.
RAPOPORT, S., Biochem. Z., 1937, *289:* 406.
ROSE, W. C., Physiol. Rev., 1938, *18:* 109.
ROSEDALE, J. L., Biochem. J., 1929, *23: 161.*
SCHMIDT, C. L. A., The chemistry of the amino acids and proteins, Baltimore, 1938.
SHARP, J. G. Biochem. J., 1939, *33:* 679.
SHARPENAK, A., O. BALSHOVA, V. MARCHENKOV, S. MENSHUTIN, M. RAVISH-SCHERBO, M. FELDT, and J. FRIEDLYAND, J. Physiol. (U. S. S. R.), 1934, *17:* 1070.
THEORELL, H., and Å. ÅKESON, Arkiv Kemi, Mineralogi och Geologi, 1943, *16 (A):* N:o 8.
THOMAS, L. E., J. K. INGALLS, and J. M. LUCK, J. Biol. Chem., 1939, *129:* 264.
VAN SLYKE, D. D., Ibidem, 1940, *133:* 287.
—, Ibidem, 1942, *146:* 150.
VAN SLYKE, D. D., and G. E. CULLEN, Ibidem, 1916, *24:* 117.
WALDSCHMIDT-LEITZ, E., and S. AKABORI, HOPPE-SEYL. Z., 1934, *224:* 187.
WEBER, H. H., Biochem. Z., 1933, *266:* 137.
WINNICH, T., J. Biol. Chem., 1942, *142:* 461.
WRETLIND, T., This Journal, 1942, *3:* 461.
ÅGREN, G., Våra Pälsdjur, 1943, In press.

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The Selection of Food.

I. General Considerations and Methodical Notes.

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It is distinctive for living substances that they maintain their internal milieu constant independently of changes in the external composition. This is effected by different kinds of regulating mechanisms which come into action as soon as the external milieu threatens the integrity of the internal milieu. As examples may be remembered those functions among the vertebrata for the maintenance of a constant osmotic pressure in the tissues. The concentration of hydrogen ions, the electrolytic composition, the glucose content etc. vary in the healthy human being within narrow limits, and if the spheres of physiological variations are exceeded, a pathological condition follows as soon as the regulatory mechanisms are unable to function satisfactorily.

Undoubtedly the absorbing of food by the organism places it in a more intimate contact with the external milieu than any other process. The organism constantly renews its material by the absorption of food. There is a continuous process of metabolism. The food constituents are taken in and absorbed to the extent to which they become micromolecular soluble in water through the digestion process. Partly they become "living" in so far as they become constituents of the cells. But sooner or later they are used up by the living substances and leave the organism, mainly in a micromolecular form. The exits are chiefly the lungs and the kidneys. Yet in spite of this continuous inflow and outflow of substances the organism retains its integrity and constant composition.

The supply of food can vary within wide limits. The organism is offered qualitatively and quantitatively various food constituents. In spite of this variation the internal milieu remains constant.

Here it must be noted that the absorption mechanism to a great extent acts without selecting as regards the substances taken per os and which either primarily or through the effect of the digestion enzymes and the gall constituents have become micromolecular soluble in water. In this respect the absorption works blindly; substances, both those useful and those poisonous for the body, which enter the intestinal canal are assimilated by the organism. Nor does there seem to be any quantitative selection: everything that passes through the intestinal canal is absorbed even if the consumed quantity of certain foods should exceed the temporary requirements of the organism.

Certain reservations as to the general validity of this rule may perhaps be made. Thus NICOLAYSEN noticed that calcium under certain test conditions is absorbed more completely by an animal (rabbit) suffering from calcium deficiency than by an animal which is completely satisfied in regard to this substance. Some observations also have seemed to prove that the absorption of iron often is more complete by an anæmic animal than by a healthy one (WHIPPLE etc.). These isolated observations ought, however, to be thoroughly tested before the assumption can be justified that the absorption mechanism itself works selectively in the sense that the intensity of the absorption adapts itself to the organism's need of the foodstuff.

On the other hand it can be definitely stated that the organism has a distinct ability to regulate any excess of food constituents received by absorption.

In this connection it is only necessary to remember the deposition of an excess of energy substances in the form of stores of foodstuffs, chiefly fat and glycogen. The secretory activity of the kidneys (and perhaps also of the intestinal canal) removes the excess of water, minerals, and other substances not needed by the body.

Even if we give full consideration to this ability of the body to organise the use of the excess of food constituents which through the absorption are incorporated in the organism's tissues, yet the *selection of food* is always the first and chief regulating factor; a regulating mechanism which is primary in cooperation with the

functions just mentioned, which above all are directed to the management of any *food excess* which has been taken. The selection of food regulates the supply of the constituents of the foodstuffs not only quantitatively but also qualitatively.

In this respect an investigation by RICHTER and HAWKES (1941) is of extraordinary interest. They allowed 45 days old rats to choose between 10 different pots of food, containing saccharose, olive oil, casein, cod liver oil, yeast and 5 different mineral salts, each in a separate pot. The animals selected from the different pots such quantities that they obtained a food composition on which they showed normal growth. The experiment thus proved in a simple way that an instinctive food selection takes place both qualitatively and quantitatively.

Many observations have been made which show that the organism is able to select certain nourishing substances to satisfy its needs. Even the single cell organism shows this ability. The classical tests were made with fern spermatozoids, after that also with bacteria. The phenomenon is, as is known, called chemo-taxis and is registered through the action of the movable micro-organisms in finding their way to the capillaries in the microscopic field which contain the substance they seek. The higher animal's effort to choose certain foodstuffs has sometimes been called "special hunger". Thus HELLWALD (1933) has measured "calcium hunger" in egg-laying hens. HARRIS and his co-workers (1933) have found that rats deprived of B-vitamin are, after some time, able to select the food which contains these vitamins. In a later communication I shall return to a discussion of these results. Finally it may be mentioned that GREEN (1925) published a report on "perverted appetites", chiefly in domestic animals. Certain diseases in them, e.g. *bovine osteofagia*, it seems possible to explain by a deficiency of some special food by means of which the animals try to satisfy their need by eating substances which they do not otherwise. Thus the disease just mentioned drives the cattle to consume bones because of their lack of phosphates.

However, most of the reports in the literature of food selection by different animals are rather primitive, and in general experiments are lacking which allow definite conclusions to be drawn, and above all it is the quantitative estimation of the choice of foods which is generally defective. A satisfactory working of this great sphere of research is badly wanted. What has been done hitherto are only experiments with a few food constituents. It

should, however, be noted that there is already a fairly comprehensive literature on the subject of feeding-up domestic animals by means of "free choice".

In this connection it seems to me important to define at the beginning, as exactly as possible, the terminology which will be used.

The observations already made seem to show that what has hitherto in scientific terminology been called "hunger" is only a collective concept, covering a series of different desires for foodstuffs. Such a desire seems to be preceded by and arise from need for foodstuffs. We need calories, water, 10 different amino acids, mineral substances and vitamins. It seems to me appropriate to reserve the word "hunger" — if it is in any case to be used as a scientific term — to the desire for calories. The need for water produces thirst. In the same way I think I can prove that the *need* for perhaps most other foodstuffs arouses a *desire* corresponding to each (the older idea "special hunger").

Later when sufficient facts have been presented we shall discuss the question of how these different desires for foodstuffs make themselves known and drive the organisms to a definite food selection.

Method.

Our method is in principle the same as that used by the majority of researchers in this subject ever since experiments were made in the chemo-taxis of micro-organisms.

It is most nearly allied to the technique of HARRIS and his co-workers (1933).

Up to the present the experiments have only been made on mice, rats, and guinea-pigs of varying ages in the different experiments. It ought, however, to be possible to use this method without inconvenience in experiments with many of our domestic animals. Several hundred animals have been used and emphasis has been laid upon basing each result on the average values obtained from as many animals as possible. In the experiments with mice 20 animals have generally been used, in those with rats and guinea-pigs about 10.

The animals are kept in individual cages. The basal diet used has varied in different experiments and will later be described in each separate case. Every day the animals were given 2 round, low, porcelain pots (about 10 cm in diameter and 2 cm in height), containing precisely the same food with the sole difference that in the one pot ("+ pot") a known concentrate of that substance was mixed whose effect was to be examined. The relative position of the pots was changed every day in order that no faulty result should be obtained by the animal forming the habit of seeking its food each day from the same position in the cage.

In contrast to that used by HARRIS and his co-workers the food was mixed with water, in most cases 70 ml water to 100 g dry food. Relatively few cases had to be rejected because of the spilling of food outside the pots. In that with rats this happened rather seldom. Mice scatter a good deal the first days after they have been taken from a mixed diet and placed on the basal diet. After some days on the basal diet, however, they generally cease to do this. Guinea-pigs like to upset the pots, so theirs must be fixed to the bottom of the cage.

Each pot is numbered on the bottom and has a correspondingly numbered counterweight. Each day the same amount of food is weighed into both pots and after 24 hours the remaining uneaten food is weighed. The food is carefully given in such quantities that the contents of the pots are never quite consumed. If that were so it is clear that the food selection is restricted and that the experiment must be rejected. As the food contains water a correction must be made for the amount of evaporation during the 24 hours. This evaporation can be estimated by determining the loss of weight in the 2—4 pots which, containing the same amount of food as in the test, are placed beside the cages. This correction is certainly not ideal because the evaporation varies with the quantity of food in the pots during the consumption period. But in the method of calculating the consumption used here and now to be described the evaporation is important as regards the reliability of the experiments only in those cases where the contents of one pot have been mainly refused.

The choice between the two pots is defined by the following quotient:

$$Q = \frac{\text{consumption from + pot}}{\text{total consumption from both pots}}$$

The quotient is a measure of the intensity of choice between the contents of the two pots and thus shows clearly the positive or negative chemo-taxis due to the contents of the + pot.

The quotient can be suitably expressed in percentage (i.e. 100 q). It can vary between the extremes 100 and 0. The figures 100—50 indicate positive chemo-taxis, the figures 50—0 negative chemo-taxis. 100 indicates that the animal has exclusively eaten from the pot with the added substance, 0 that the added substance has driven the animal wholly to refuse this food. The quotient 50 indicates no selection. This figure was also obtained regularly if both pots had exactly the same contents and the experiment series comprised a sufficient number of animals. The variations around 50 have proved to be in a definite relation to the concentration of the added substance in the food. In this way the quotient is a peculiarly suitable and extremely sensitive numerical expression for the animal's selection of food.

Yet in judging these quotients several factors must be taken into account which have not had sufficient consideration in earlier experiments.

Clearly the taxis can be tested either by adding chemically pure substances to the contents of the +pot or by the addition of mixed foods.

When chemically pure preparations are added the judging is usually relatively simple, presuming that the substances are really so pure that traces of, for example, the organic solvents used in preparing the substance or other impurities do not give the food a specific smell or taste. As, however, many foodstuffs, e.g. synthetic vitamins, only require to be tested in concentrates forming only fractions per 1,000, it is considered that impurities amounting to some tenths of one % can scarcely have any great effect as they will occur in the food in such infinitesimal concentrations.

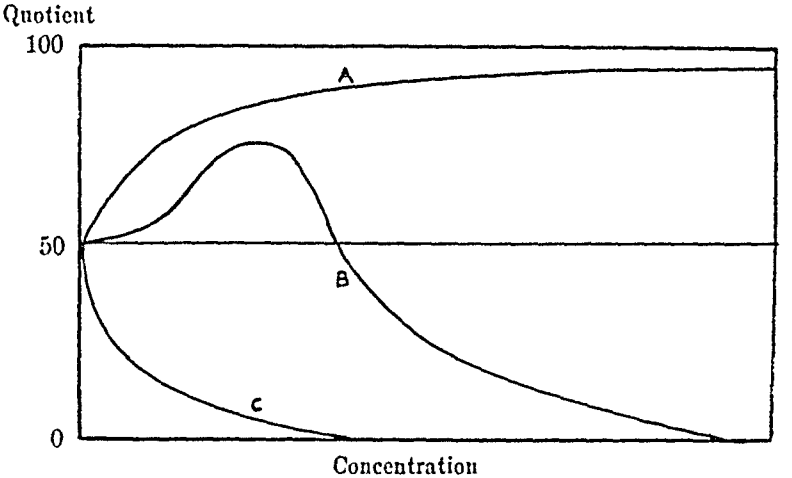

Fig. 1.

Thus, presuming that the added chemical substances are sufficiently pure, the quotient gives a direct and reliable figure of the positive or negative chemo-taxis due to the substance in question. Such quotients can be reproduced to an unlimited extent in repeated experiments assuming that the animal's condition, age, sex and food concentrates are similar.

In proportion to the concentrations a series of different types may arise. These can be illustrated graphically as in fig. 1, where the ordinate represents the quotient values and the abscisse the concentrations of the tested substance:

A, after the passage of the minimum perceptibile there arises at a higher concentration a positive chemo-taxis which asymtotically approaches 100 in all the tested concentrations. (If the concentrations are sufficiently high it is clear that the curve will be of the type B.):

B, after the passage of a minimum perceptibile a positive chemo-taxis arises at certain concentrations which is transformed into a negative chemo-taxis if the concentration becomes higher:

C, the substance produces only a negative chemo-taxis, possibly after passing through an area below the minimum perceptibile.

It is specially important to emphasise that a positive chemotaxis for a foodstuff often only appears when there is a deficiency of it in the organism. Fully-nourished, and in certain respects undernourished, animals show different taxes (Cf. what was said above of the relation between need and desire.). These are only some examples of the types which may represent the results of testing pure substances in different concentrations.

In testing mixtures, e.g. vitamin preparations, their concentrates, food substances, spices, flavourings, etc., more complicated conditions arise which demand special consideration.

It must be assumed that these mixtures may contain some substances which exert a positive chemo-taxis, and some which exert a negative chemo-taxis.

Thus, a quotient under 50 can be observed if the mixture contains one or more unattractive substances together with the one which is the object of the investigation; the animal refrains from eating the food although it contains a substance of which it is in need, and which under other conditions, e.g. mixed in a chemically pure form, would give a quotient over 50. Most careful regard must be paid to these different eventualities in judging the results of the experiments.

Summary.

1) Food selection both qualitatively and quantitatively is the chief regulatory action in preserving the integrity of the organism in relation to the external milieu.

2) The *need* for food has been called starvation, hunger, thirst, special hunger etc. The same concept has been used to indicate *desire* for food. The author wishes to introduce a more closely defined terminology by sharpening the difference between the *need* of foodstuffs and the *desire* for foodstuffs. The former implying that certain forms of luxury consumption are here ignored, — the necessary presumption for the rise of the latter.

3) Experiments have already been made which indicate that in the animal organism certain desires for food of different kinds arise, corresponding to the need for the different foods. Thus, "hunger" is a collective concept which can be resolved into a whole series of needs for food with corresponding desires for food, leading to different food selection for the satisfying of the different needs. Thus "thirst" is desire for H_2O. The author proposes that in

the scientific literature of this subject the word "hunger" should be reserved for the desire for calories, which arises when a supply of energy is needed.

4) The discussion of the mechanism of the desire for food, general feelings, sensations of taste, instinctive impulses is postponed until the author presents his observations in later communications.

5) The method used by the author for the measuring of food selection by animals — rats, mice and guinea-pigs — is described in detail. The interpretation of the quantitative facts obtained by this method is discussed.

References.

GREEN, H. H., *Physiol. Rev.* 1925. *5.* 336.

HARRIS, L., J. CLAY, F. J. HARGREAVES, and A. WARD, Proc. Roy. Soc. Lond. Ser. B. 1933. *113.* 161.

HELLWALD, H., Untersuchungen über Treibstärken bei Tieren. Diss. Rostock 1933.

RICHTER, C. P. and C. D. HAWKES, Amer. J. Physiol. 1941. *131.* 639.

From Universitetets biokemiske Institut, Copenhagen.

On the Effect of Cyanide on the Respiration of Yeast.

By

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Received 4 December 1943.

Introduction.

The study of the effect of cyanide on cell respiration has been of the greatest importance in the development of the enzymic chemistry of biological processes. This is brought out particularly clearly by the investigations of O. WARBURG and his coworkers. The importance of cyanide in this connection is due to its property of forming complexes with heavy metals, and thus of inhibiting enzyme systems which are connected in some way or other with heavy metal catalysis.

However, a survey of the literature shows that the problems connected with cyanide inhibition of respiratory processes are probably far more complex than was at one time thought. We cannot here give an exhaustive discussion of all the relevant investigations, but must restrict ourselves to a brief recapitulation of some of the more striking results. (A complete bibliography can be found in, for instance, OPPENHEIMER (1939)).

It is of interest first to observe that KISCH (1933) has found a rather strong increase in the respiration of certain animal tissues by the addition of cyanide in very small concentrations (less than m/20,000). His work was done on rat kidney slices. So far no complete and coherent explanation of these results seems to have been put forward. The effect found by KISCH seems to

have found a certain analogy in a discovery made by Cozic (1934/36) in her work on bacteria of the Acetobacter group. She found, on investigating the respiration of these organisms in the ordinary way in a WARBURG apparatus, that while the respiration of all the other Acetobacter species was inhibited by cyanide, as one is accustomed to find in animal tissues, one particular species, namely Aectobacter xylinum, showed a great increase in respiration (up to 5 to 10 fold) at cyanide concentrations up to m/10 KCN. No definite explanation could be given for this quite unprecedented behaviour, but COZIC tentatively advanced the hypothesis that the effect is due to some reaction of the cyanide with the cellulose membrane of the bacteria in question. This idea seems to be of a very hypothetical nature, and it can probably safely be said that it is neither supported nor disproved by our present very incomplete knowledge of the cell wall in bacteria.

In this connection we may also mention some similar effects observed with hydrogen sulphide and carbon monoxide. The action of these two compounds on cell respiration is in general quite similar to that of cyanide, the underlying mechanism being analogous in the two cases. However, NEGELEIN (1925) in his work on Chlorella found that the respiration of this organism may be increased up to 80 per cent under the action of hydrogen sulphide. Carbon monoxide can also, under certain conditions bring about a very marked increase of cell respiration. Thus we may mention the experiments of FENN (1932), who worked with certain muscle preparations, and of BODINE c. s. (1934, 1936, 1937), who studied the embryonic development of Melanoplus. Other work on this problem is due to SCHMITT and SCOTT (1934), and ÖRSTRÖM (1935). These experiments on carbon monoxide may, after all, not be so difficult to understand, since, according to the work of NEGELEIN (1931), carbon monoxide may be oxidised catalytically in the presence of hemines.

The purpose of the present note is to report upon some experiments, which, in a certain analogy with some of the work discussed above, seem to reveal some interesting activating effects of cyanide on the respiration of yeast. It seems to us that the explanation of these effects are at present rather obscure, but in spite of this we have thought that the experiments may be of sufficient interest to justify their being put on record.

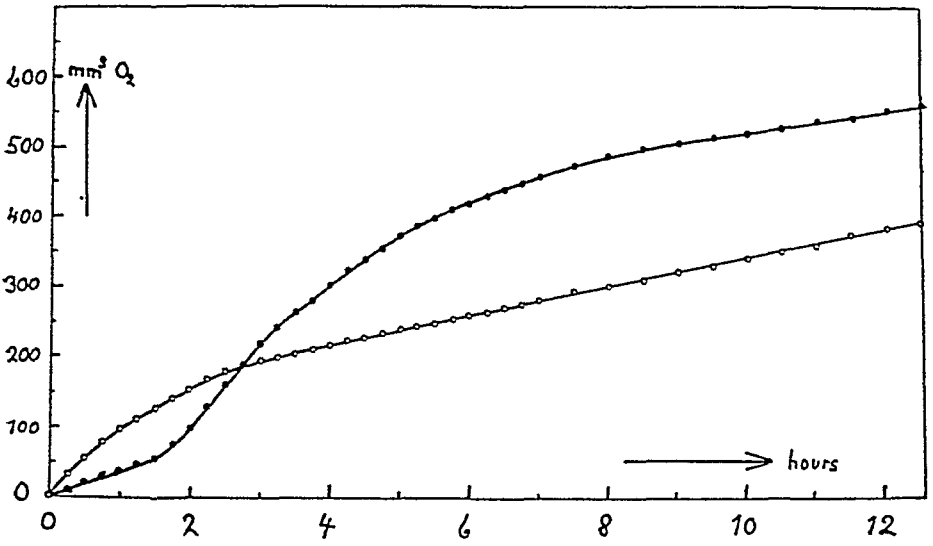

Fig. 1.

○———○ Oxygen uptake of yeast suspended in 3 ml. phosphate buffer of pH 7.28.

●———● Oxygen uptake of yeast suspended in 2 ml. phosphate buffer of pH 7.28, with the addition of 1 ml. of m/10 KCN of the same pH.

Experimental Part.

The respiration of yeast was studied in the ordinary way by measuring its oxygen uptake in a Warburg apparatus. Ordinary baker's yeast was used throughout, suspended in phosphate buffer of pH around 7.3; the cyanide solution was neutralized and brought to the same pH as the buffer. For absorption of CO_2 the central chamber of the vessel contained 0.2 ml. of a strong solution of potassium hydroxide. The total amount of buffer solution used was usually around 3 ml. The volume of the Warburg vessels was around 17 ml. The temperature in the water bath was 38° in all experiments.

Some of the results are shown in the diagrams in figs. 1—2. These curves show the oxygen uptake as a function of time for yeast with and without addition of cyanide. They show uniformly that when cyanide is added we have to begin with a strong inhibition of the oxygen uptake. After some time, however, the oxygen consumption suddenly increases strongly, and rises considerably above the value obtained for yeast without any cyanide.

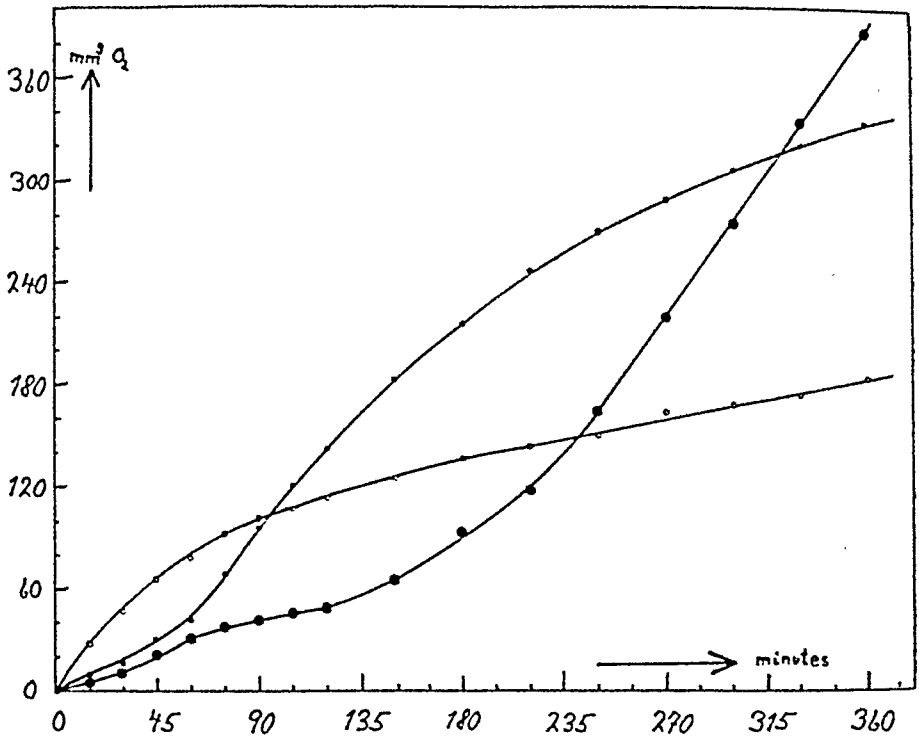

Fig. 2.

○——○ Oxygen uptake of yeast suspended in 3 ml. phosphate buffer of pH 7.28.

●——● Oxygen uptake of yeast suspension in 2 ml. phosphate buffer of pH 7.28, with the addition of 1 ml. m/100 KCN of the same pH.

●——● Oxygen uptake of yeast suspended in 2 ml. phosphate buffer of pH 7.28, with the addition of 1 ml. m/10 KCN of the same pH.

As is shown by the diagrams, the rise in oxygen consumption occurs later with increasing cyanide concentration, but the rise becomes steeper. This behaviour was found to be a common feature of all our experiments.

Fig. 3 shows the results obtained when glucose was added to the yeast suspension. In this case no sudden rise in the oxygen uptake was observed; as the diagrams show we found just uniform inhibition by cyanide.

Attempts at finding similar effects for other cells, such as bottom yeast, and rat liver and brain tissue, failed.

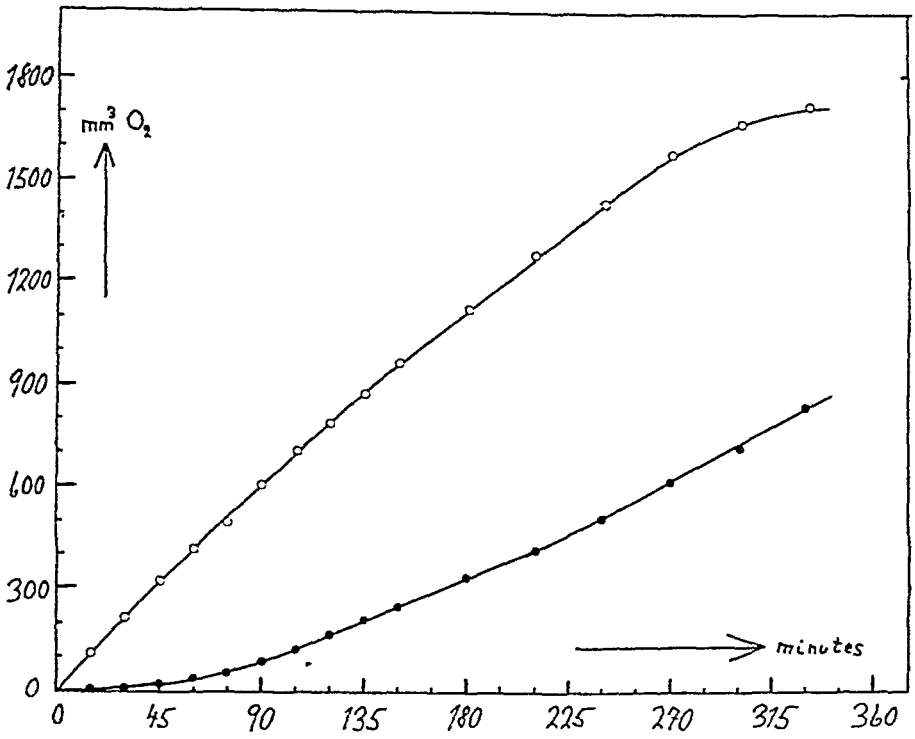

Fig. 3.

○———○ Oxygen uptake of yeast suspended in 3 ml. phosphate buffer of pH 7.28 and with 0.1 % glucose.
●———● The same, but containing m/10 KCN.

Discussion.

As already mentioned we do not see any immediate and obvious explanation for the results described above, although a simple interpretation possibly exists. It seems reasonable to assume that such an explanation is to be found in the interference of the cyanide with the various enzymic systems that contribute to or participate in the cell respiration. However, the conditions are so complex that it is far from easy to analyze the various factors separately. We shall here restrict ourselves to a brief indication of some features of the experimental results reported above, which may have a particular bearing on their ultimate explanation. In this connection we mention first the fact that a certain time must always elapse before the onset of the increase in oxygen uptake. It seems therefore that the effects described

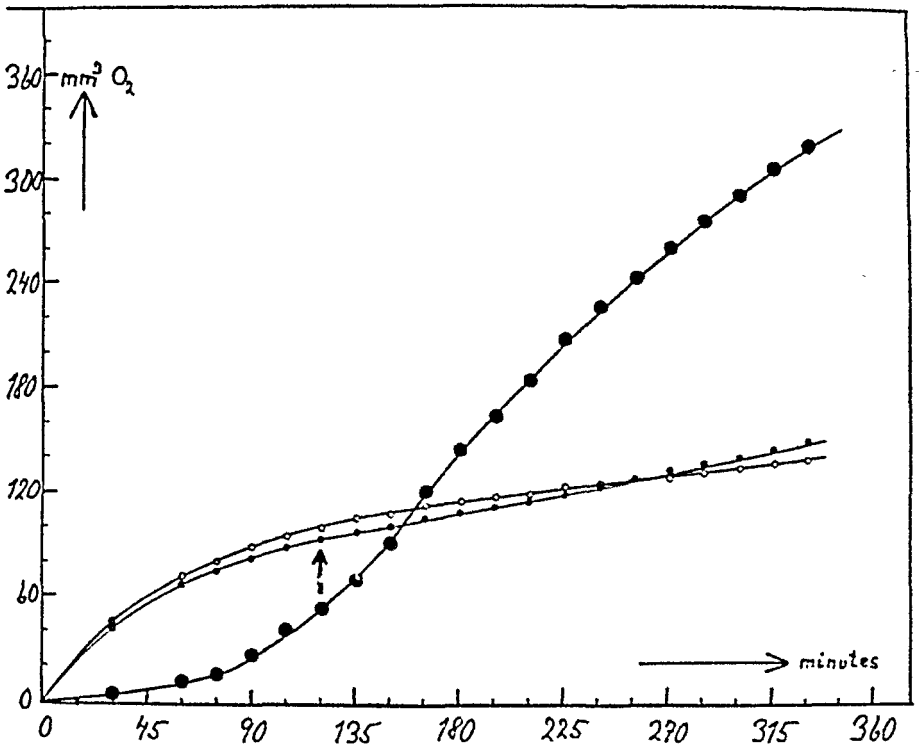

Fig. 4.

○———○ Oxygen uptake of yeast suspended in 3 ml. phosphate buffer of pH 7.28.

●———● Oxygen uptake of yeast suspended in 2 ml. phosphate buffer; 1 ml. m/10 KCN added at the point indicated by the arrow.

●———● The same with 1 ml. m/10 KCN added at the beginning.

only occur after an initial depletion of some material originally present in the yeast. It may be important in this connection that, as shown above, the presence of glucose in the medium prevents the occurrence of the effect. Finally we may also mention that in an experiment, the results of which are shown in fig. 4, and in which cyanide was added after the lapse of a certain time, no effects were observed; thus it seems that the cyanide has to be there from the beginning.

On the basis of the well-known fact that the CN-group reacts with aldehydes and ketones to form cyanhydrins, it might be thought that the effect depends in some way or other on a reaction of the cyanide with intermediary metabolic products, or perhaps enzymes of intermediary metabolism, containing carbonyl groups. This assumption, however, does not appear very

probable, since semicarbazid was found to have no effect on the respiration.

One might therefore rather think that the effect is connected with the other characteristic property of the CN-group, i. e., that of forming complexes with heavy metals. Observations have been recorded in the literature showing that the inhibition of catalase may under certain conditions lead to an increased oxygen uptake, but whether this is sufficient to explain all the effects reported above are not quite clear to us yet and a final decision is therefore postponed for the moment.

In closing we may perhaps mention that from a purely logical point of view one might at first make the — admittedly extremely improbable — hypothesis, that the cyanide was oxidized in some way or other through the mediation of the yeast cells, and that this might account for the extra oxygen consumption. Such an assumption, however, is directly contrary to all other experience in this field, and was, moreover, easily refuted by some simple colorimetric determinations of the concentrations of cyanide in solutions with or without yeast cells.

Summary.

After an introductory review of the activating effects of cyanide on cell respiration described in the literature, the authors report a similar effect with yeast cells, which under certain conditions show a strong increase in oxygen uptake under the action of cyanide. The possible interpretations of the phenomenon are briefly discussed.

References.

BODINE, J. H. c. s., J. cell. physiol. 1934, *4*, 397, 475, *5*, 97.
—, Proc. nat. Acad. Sci., Wash. 1934, *20*, 640.
—, J. cell. physiol. 1936, *8*, 213.
—, Proc. Soc. exp. Biol., N. Y., 1937, *36*, 21.
COZIC, J., Rev. gen. bot. 1934—36, *46*, 1, *48*, 209.
ELLIOTT, K. C., Handb. d. Katalyse 1940—41, *3*, *292*.
FENN, W. O., Amer. J. Physiol. 1932, *101*, 34, *102*, 393.
KISCH, B., Biochem. Z. 1933, *263*, 187.
NEGELEIN, E., Ibidem 1925, *165*, 203.
—, Ibidem 1931, *243*, 386.
OPPENHEIMER, C., Supplemente, *1938*, 1265.
SCHMITT, F. O, and M. G. SCOTT, Amer. J. Physiol. 1934, *107*. 85.
ÖRSTRÖM, A., Protoplasma 1935, *24*, 177.

From the Biological Institute of the Carlsberg Foundation, Copenhagen.

On the Employment of Buffers of Constant Ionic Strength in Enzyme and Protein Chemistry.

By

ROLF BRODERSEN.

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In enzyme and protein chemistry buffers are employed extensively in studies on the influence of changes in the hydrogen ion concentration. A change in the hydrogen ion concentration of a buffer naturally implies a change in the concentrations of the other components of the buffer. These changes differ in character with a given change in hydrogen ion concentration according to the buffer composition used, and will influence the results obtained with the buffer.

ASTRUP (1942) has investigated the action of thrombin on fibrinogen and found the velocity of this process to depend both on the hydrogen ion concentration and on the composition (ionic strength) of the buffer. In biochemical investigations, therefore, it is necessary also to take into account the composition of the buffer employed.

Previously a number of different formulae have been described for the composition of buffers. The acetate buffer of SORENSEN (1909) has a constant total molarity of acetic acid and sodium acetate. MICHAËLIS (1913, 1931) describes buffers of constant ionic strength. This principle has found employment in the protein chemistry; (see, for instance, TISELIUS (1937) and to a limited extent in the chemistry of enzymes (ASTRUP, 1942). In physical chemistry dilute buffers are used in relatively concentrated salt solutions (BRØNSTED, 1921).

The following studies will show how the buffers have to be composed when it is desirable, as far as possible, to eliminate the effect of changes in all concentrations other than that of the hydrogen ion concentration.

Principle of the Method employed.

In order to investigate the influence of changes in the concentration of compounds other than hydrogen ions it is preferable to use a system with properties independent of the hydrogen ion concentration, but highly dependent on the other qualities of the buffer that may eventually influence the physico-chemical properties of enzymes and proteins. These conditions are met by the ferro-ferricyanide electrode, which consists of a platinum wire in a solution containing potassium ferrocyanide and potassium ferricyanide. In this, the following equilibrium is established:

$$Fe(CN)_6^{----} \rightleftharpoons Fe(CN)_6^{---} + e^-$$

The potential of the electrode will be:

$$\pi = \frac{RT}{F} \ln \frac{[Fe(CN)_6^{---}]}{[Fe(CN)_6^{----}]} \cdot \frac{f^{---}}{f^{----}} + \pi_0,$$

where f is the activity coefficient.

This potential is largely dependent on the salt composition of the medium, as the two activity coefficients are highly dependent on the salt content and to a different degree. On the other hand the potential is independent of the hydrogen ion concentration or, rather, its dependency on the hydrogen ion concentration does not differ essentially from its dependency on the concentration of other ions in not too acid solutions.

So, when buffers of different hydrogen ion concentration are added to the solution, a constant potential will be obtained if the salt effect of the buffer does not also vary with its hydrogen ion concentration. If it does, the change in salt effect will alter the potential, unless the effects on the two activity coefficients would counterbalance each other — a rather improbable possibility.

Presumably the amount of uncharged components in the buffer is of smaller significance of the state of the protein dissolved in it in comparison with the amount of salt. Nor have uncharged

substances any particular influence on the potential of the ferro-ferricyanide electrode.

In these considerations no account is taken of the liquid junction potential at the connection with the calomel electrode. Whether the measured changes be due to variations in this potential or in the electrode potential is not of decisive importance to the conclusion. For the ideal buffer must also meet this requirement: that it causes no change in the liquid junction potential simultaneously with a change in hydrogen ion concentration. As the liquid junction potential and the electrode potential cannot be measured separately, there is no reason to distinguish between these effects.

The method is not useful with p_H lower than 4—5, as the complex ions employed are stable only at low hydrogen ion concentrations. Furthermore, of course, the ions must not react chemically with the components of the buffers examined.

Experimental.

The concentration of both potassium ferrocyanide and potassium ferricyanide employed was 0.001 m, which proved to give potentials reproducible with an accuracy exceeding 0.1 mV. The determinations were carried out in a water-thermostat at 22°, as the potentials proved to be highly dependent on the temperature. As a standard electrode a saturated calomel electrode was employed. The small variations in the potential of this electrode were eliminated by occasional reedings of the potential of the cell:

Pt	0.001 m $K_3Fe(CN)_6$ 0.001 m $K_4Fe(CN)_6$	saturated KCl » Hg_2Cl_2	Hg

and subtracting this from the potential of the cell

Pt	0.001 m $K_3Fe(CN)_6$ 0.001 m $K_4Fe(CN)_6$ Buffer	saturated KCl » Hg_2Cl_2	Hg

This gives the potential of the cell

Pt	0.001 m $K_3Fe(CN)_6$ 0.001 m $K_4Fe(CN)_6$ Buffer	saturated KCl	0.001 m $K_3Fe(CN)_6$ 0.001 m $K_4Fe(CN)_6$	Pt

This potential is a direct measure for the salt effect of the buffer on the system here employed.

Addition of 2 % saccharose (0.1 m) changes the potential merely 1 mV. On addition of 0.1 m salt (*e. g.*, NaCl) the change in potential is considerably larger (33 mV).

For the sake of illustration, the results obtained for sodium phosphate buffer are given in Table 1.

Table 1.

$\frac{[NaH_2PO_4]}{[Na_2HPO_4]}$	P_H calculated $p_H = p_K - \log \frac{[NaH_2PO_4]}{[Na_2HPO_4]}$	m const. = 0.1 $= [NaH_2PO_4] + [Na_2HPO_4]$	μ const. = 0.1	
			Undiluted $\mu = [NaH_2PO_4] + 3[Na_2HPO_4]$	Diluted 1 : 10 with 0.1 m NaCl
		mV	mV	mV
9 : 1	5.75	32.4	29.3	32.9
8 : 2	6.10	33.4	28.5	32.8
7 : 3	6.33	34.9	27.7	32.7
6 : 4	6.52	35.8	27.3	32.7
5 : 5	6.70	36.8	27.0	32.7
4 : 6	6.88	37.8	26.5	32.7
3 : 7	7.07	38.7	26.3	32.5
2 : 8	7.30	39.9	26.1	32.5
1 : 9	7.65	40.4	26.3	32.5

The results obtained are presented graphically in Fig. 1. The full-line curve (NaCl 0.1 m) gives the potential after the addition of 0.1 m NaCl. This potential is not altered by addition of small amounts of a strong acid or base. The points ○ indicate the results obtained for some of the classical buffers of varying ionic strength, while the points □ correspond to constant ionic strength. None of these buffers met the stipulated requirements.

The values represented by the points △ were obtained by means of buffers (citrate and phosphate with constant ionic strength of 0.1, and borate after SØRENSEN) diluted 1 : 10 with 0.1 m sodium chloride. They lie approximately on a straight line parallel to the abscissa, and not far below the potential obtained in a 0.1 m NaCl solution. From Fig. 1 it will be noticed that the distances from corresponding points of the curve for

0.1 m sodium chloride to respectively the curve for NaCl-containing buffer and the curve for NaCl-free buffer are as 1 : 10, that is, as the degree of dilution with 0.1 m sodium chloride. It is reasonable to expect, then, that higher dilution would have resulted in an even better approximation to the curve for 0.1 m sodium chloride.

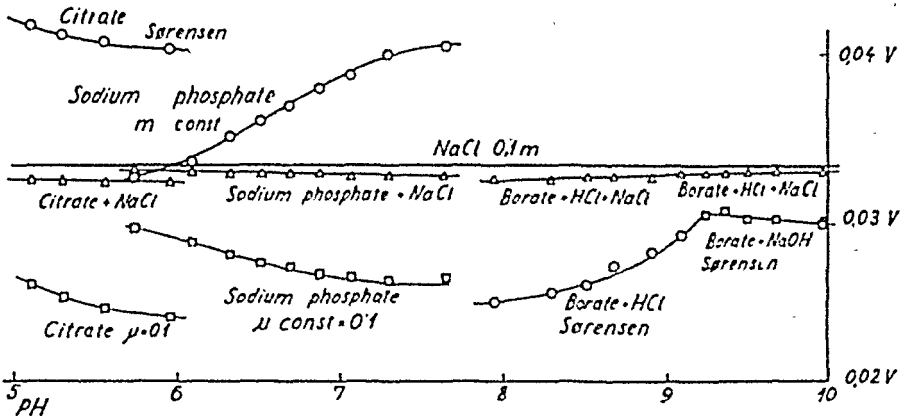

Fig. 1.

Discussion.

As far as this system is concerned, then, neither buffers of constant total molarity nor constant ionic strength are suitable when the question is to investigate the effect of changes in the hydrogen ion concentration. Only mixtures of the buffer in low concentration with sodium chloride in higher concentration are suitable.

Something similar may be expected to apply to proteins and enzymes. In some cases, however, it is sufficient to keep the ionic strength constant, while it is immaterial which ions are present (TISELIUS, 1937). Still, the degree of dilution of buffers generally employed in enzyme and protein chemistry is far from sufficient always to make the ionic strength constitute the only decicive factor. Indeed, salt effects of the employed buffer have been observed which could not be ascribed to the ionic strength. In studies on enzymatic cleavage of glucosides, VEIBEL (1940) found the p_H optimum to differ in different buffers. Also the magnitude of the maximal reaction velocity depends on the character of the buffer. These differences are found even when the ionic strength is kept constant from one buffer to another.

DAVIS and COHN (1939) have investigated the dependence of the mobility of carboxyhemoglobin on the hydrogen ion concentration in citrate and phosphate buffers of different ionic strength, and found it to depend not only on the ionic strength but also, in a marked degree, on the nature of the ions. By interpolation the isoelectric point is determined at each ionic strength examined. As was to be expected, the curves for the dependency of the isoelectric point on ionic strength in phosphate and buffers approach each otner with decreasing ionic strength, presumably intersecting at zero ionic strengtn.

Conclusion.

For examination of the effect of changes in the hydrogen ion concentration on phenomena in enzyme and protein chemistry it is necessary to employ a diluted buffer in a relatively concentrated salt solution. The concentrated buffers generally employed cannot be expected to give reliable results — not even when constant ionic strength is used.

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I am greatly obliged to Dr. GÜNTELBERG, Ph. D., for advice during this work.

References.

ASTRUP, T., Biochem. Z. 1942, *313*, 229.
BRONSTED, J. N., J. Chem. Soc. 1921, *119*, 574.
DAVIS, B. D. and E. J. COHN, J. Amer. Chem. Soc. 1939, *61*, 2092.
MICHAËLIS, L., Biochem. Z. 1931, *234*, 139.
—, und A. MENDELSOHN, Biochem. Z. 1913, *58*, 315.
SØRENSEN, S. P. L., C. R. Lab. Carlsberg 1909, *8*, 1.
TISELIUS, A., Biochem. J. 1937, *1*, 313.
VEIBEL, S., Enzymologia 1940, *9*, 161.

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On the Respiration of the Reticulocytes in Relation to the Ripening.

By

ERIK JACOBSEN and CLAUS MUNK PLUM.

Received 7 December 1943.

It is known that unripe red blood cells have a larger oxygen consumption than the mature blood cells. Although they have no nucleus, the reticulocytes too possess a larger metabolism in comparison with the non-reticulated erythrocytes. Owing to this fact blood from animals made anemic through blood lettings or through treatment with poisons such as phenylhydrazine hydrochloride has been extensively used in studies on the oxygen metabolism of the red blood corpuscles. Among papers on this problem we shall only refer to those of WARBURG (1914) principally dealing with the influence of narcotics and other inhibiting substances and of WRIGHT (1931) who mainly studied the influence of various salt concentrations, the adding of glucose and amino-acids, and the influence of pH.

WRIGHT finds that 100 cmm reticulocytes from rabbits made anemic with phenylhydrazine hydrochloride have an oxygen consumption of about 70 cmm per hour, while 100 cmm nucleated normal red blood cells from fowl only have an oxygen comsumption of 12 cmm per hour. From this he concludes that the reticulum rather than the nucleus appears to be closely associated with the cell oxidation either as a material consumed or as the mechanism by means of which the process is effected.

The reticulocytes are cells in development. They represent a stage between the erythroblasts, the nucleated cells of the bone marrow, and the fully matured erythrocytes normally circulating in the blood vessels. Both in vitro and in vivo the reticulocytes

ripen into normal red blood cells, and as the normal red blood corpuscles in mammals have practically no uptake of oxygen, it seems natural to presume some connection between the ripening processes and the oxygen consumption in the blood cells.

Recently it has been shown that the ripening of the reticulocytes is influenced by some factors found in liver extracts and in blood plasma (PLUM 1942). Reticulocytes suspended in saline ripen very slowly, while an addition of liver extract to the saline accelerates the ripening process considerably, five to ten times the ripening rate in the saline or even more, depending on the concentration of the ripening substances in the medium. As to the possible physiological significance and the chemical character of the ripening substances we may refer to the papers of PLUM (1942 a, b, 1943), and of JACOBSEN and PLUM (1942). After the discovery that external factors thus influence the ripening of the blood cells, several problems arise. The present paper deals with the relation between the ripening and the oxygen metabolism of the reticulocytes. In some experiments we have varied the ripening intensity by the addition of ripening substances and studied the effect on the oxygen comsumption, and in others we have varied the oxygen consumption by the addition of various drugs known to have a specific influence on the cell metabolism, and studied the effect on the rate of ripening.

Technique.

The blood cells used were from rabbits made anemic by constant blood lettings. 5 ml blood were centrifuged, the plasma sucked off, and the blood cells were suspended in 5 ml of the liquid to be examined (saline, saline with liver extract with or without other substances added). 2 ml of the thoroughly mixed suspension were used for ripening experiments, while another 2 ml were placed in the WARBURG vessels for determination of the oxygen uptake. Both were examined at 40°. The oxygen consumption was controlled during the whole time in which the ripening experiments were continued, mostly six hours. The ripening experiments were carried out as described by PLUM (1942 a, b) and JACOBSEN and PLUM (1942). The rate of ripening is here expressed through the monomolecular constant, as in all previous papers on the same subject.

Results.

1. The Ripening Rate and the Respiration.

All the experiments made on the respiration of reticulocytes suspended in saline or in saline to which ripening substances had

been added gave the same results. The ripening rate can be augmented several times without increase in the oxygen consumption.

Table 1 shows such an experiment.

Table 1.

In each vessel 2 ml blood corpuscles with 205 ‰ reticulocytes suspended in saline or saline with liver extract (Hepsol fortior).

Suspended in	cmm oxygen consumed after				Monomolecular constant of ripening velocity
	½ hour	2 hours	4 hours	6 hours	
Saline	28	103	150	188	0.0132 (k_s)
Saline with 1 % liver extract .	29	106	153	175	0.0356 (k_f)
Saline with 2 % liver extract .	18	93	132	155	0.0574 (k_f)

There is a considerable decrease in the rate of respiration during the experiment. A calculation shows that this cannot be explained through the decline in the number of reticulocytes. In the vessel containing saline the oxygen consumption in the first half hour is 28 cmm with 205 ‰ reticulocytes present, while the consumption in the last half hour is only 8 cmm with 172 ‰ reticulocytes. It is impossible to say whether this decline in oxygen consumption is due to the using up of the donator substances present in the blood corpuscles or to the destruction of enzymes or carrier systems.

2. The Effect on the Ripening Rate of Factors Influencing the Respiration.

These experiments have at times presented some difficulties. The red blood corpuscles often lose their structure when the oxygen metabolism is arrested for some length of time. The reticulocytes stain badly and a considerable hemolysis occurs. Even after 2—4 hours' incubation in a nitrogen atmosphere this may happen. Therefore several of the experiments could not be continued for more than four, in some cases two hours. This of course does not give the same certitude in the ripening experiments as usual, but the influence is so marked that the results must be considered beyond doubt.

a. Influence of the Oxygen Pressure.

Table 2 shows the results of a typical experiment.

Table 2.

Influence of oxygen pressure on the respiration and ripening of reticulocytes.

Reticulocytes in saline with 2 % liver extract.

% Oxygen in N_2	cmm O_2 consumed after the first hour	Monomolecular constant of ripening rate
20 %	36	0.0606
10 %	31	0.0516
5 %	27	0.0457
0 %	—	0.0406

It is evident that reduced oxygen pressure reduces the ripening rate. The ripening is, however, not totally arrested; in several experiments where the blood was kept under strictly anerobical conditions — evacuated and under alkaline pyrogallol — the ripening constant was about half that of blood kept under atmospheric air in the time it could be followed before the hemolysis occurred.

Table 3.

Influence of ethyl-urethane on respiration of ripening reticulocytes.

Reticulocytes suspended in	cm O_2 consumed after		Monomolecular constant of ripening rate
	2 hours	6 hours	
Saline	61	116	0.0135
Saline with m/8 ethyl-urethane	33	57	0.0109
Saline with m/4 ethyl-urethane	29	50	0.0076
Gastric extract with 0.1 ‰ Tyrosin . .	57	105	0.0596
Gastric extract with 0.1 ‰ Tyrosin m/8 ethyl-urethane	25	81	0.0436
Gastric extract with 0.1 ‰ Tyrosin m/4 ethyl-urethane	29	58	0.0323

m/2 ethyl-urethane gave hemolysis in all vessels.

b. Influence of Narcotics.

WARBURG (1914) has shown the inhibiting influence of narcotics on the red blood-corpuscles. We have used two urethanes, ethyl-urethane and butyl-urethane "Hedonal"; both had a marked influence on the ripening rate (Table 3).

As to the butyl-urethane 0.025 m Hedonal gave a respiration of 35 p. c. and a ripening rate of 70 p. c. of the normal values. The corresponding figures for 0.010 m were 70 % of normal respiration and 85 % of normal ripening. 0.05 m caused hemolysis already after 2 hours' incubation.

d. Influence of Fumarate, Oxalacetate and Malonate.

During their investigations on the rôle of the four-carbonatom-dicarboxylic acid in tissue respiration SZENT-GYÖRGYI and al. (1935) found that the addition of fumarate or oxalacetate to respirating tissues is able to stimulate the oxygen consumption. The oxygen uptake of tissue is moreover greatly inhibited by malonic acid which inhibits the succinodehydrogenase in the same concentrations. The inhibition of malate on cellular respiration is completely abolished by the addition of fumarate (or oxalacetate). The experiments of SZENT-GYÖRGYI and co-workers were mainly carried out with minced pigeon-breast muscle but our experiments show that the same can be seen when reticulocytes are used as a respiring system. As to the ripening rate it closely follows the variation in oxygen uptake after the addition of fumarate, oxalacetate, malonate and malonate plus fumarate, the effects of which correspond to the abovedescribed (Tables 4 and 5).

SZENT-GYÖRGYI concludes from his experiments that the systems malate $\rightleftarrows$ oxalacetate and succinate $\rightleftarrows$ fumarate act as hydrogen carriers. His theories have been modified several times and for details we refer to the originals. The finding can however, also be explained from the citric acid cycle theory of KREBS. It is beyond the scope of this paper to discuss the two theories but it is evident that in this respect the red blood cells behave like the muscle tissue hitherto examined, and that the ripening processes follow the rate of respiration. One of the collaborators of SZENT-GYÖRGYI, I. BANGA (1936) has moreover shown that oxalacetate added to certain respiring tissues for a

Table 4.

Effect of Fumarate and Malonate on ripening rate and respiration.

Reticulocytes suspended in	cmm oxygen consumed in		Monomolecular constant of ripening rate
	1 hour	6 hours	
Saline	53	148	0.0139
Saline with 0.0025 m malonate	40	108	0.0129
Saline with 0.0025 m fumarate	54	163	0.0142
Saline with 0.0025 m malonate and 0.0025 m fumarate	50	144	0.0141
Saline with 2 % liver extract	55	152	0.0708
Saline with 2 % liver extract and 0.0025 m malonate	38	107	0.0475
Saline with 2 % liver extract and 0.0025 m fumarate	61	171	0.0803
Saline with 2 % liver extract and 0.0025 m malonate, 0.0025 m fumarate	51	147	0.0722

Higher concentrations could not be used without hemolysis. Curiosly enough, oxalacetate could be tolerated in a much higher concentration.

Table 5.

Effect of Oxalacetate on ripening index and respiration.

Reticulocytes suspended in	cmm oxygen consumed in		Monomolecular constant of ripening rate
	1 hour	6 hours	
Saline	45	146	0.0142
Saline with 0.01 m oxalacetate	75	198	0.0154
Saline with 2 % liver extract	40	139	0.0718
Saline with 2 % liver extract and 0.01 m oxalacetate	77	200	0.0916

short time is able to arrest the oxygen uptake, while it is reduced to malate, and thus competes with the oxygen as a hydrogen acceptor. This happens when muscle, liver and kidney tissue is used as a respiring medium while embryonic, lung and tumor tissue is not able to reduce oxalacetate. Our experiments show that the reticulocytes belong to the first category of tissues able to reduce oxalacetate. It is to be regretted that it is impossible to

arrest the oxygen uptake with oxalacetate for so long that the ripening process can be studied during this condition, as this requires a concentration of oxalacetate which causes hemolysis.

e. Influence of Cyanide and Carbonmonoxide.

As is well known, the inhibiting effect of cyanide and carbon monoxide on the oxygen uptake is caused by a specific inhibition on the last link of the hydrogen-carrying chain, the cytochrom oxydase.

In the reticulocytes both these inhibitors have an effect on the respiration as well as on the ripening.

Table 6 shows the effect of cyanide and Table 7 that of carbonmonoxide.

Table 6.

Effect of Cyanide on respiration and ripening index.

Reticulocytes suspended in	cmm oxygen consumed in		Monomolecular constant of ripening rate
	1 hour	6 hours	
Gastric extract with 0.1 ‰ Tyrosine . .	44	124	0.0611
Gastric extract with 0.02 m KCN . . .	22	61	0.0331
Rabbit plasma	38	156	0.0310
Rabbit plasma with 0.02 m KCN	23	82	0.0187

When suspended in saline cyanide concentrations high enough to give a marked inhibition of the respiration caused a hemolysis which made it impossible to make ripening experiments. The concentration of cyan should, however, be astonishingly high to cause any inhibition; in the above-mentioned experiment 0.008 m KCN gave only a slight decrease (3—16 p. c.) in respiration and ripening rate.

During the incubation all the vessels in this experiment were kept in the dark. It is one of the merits of WARBURG to have shown that light abolishes the inhibition of carbonmonoxide on respiration. It can be shown that the light has an effect not only on respiration in a carbon monoxide atmosphere but on the ripening rate as well (Table 8).

Table 7.

Effect of Carbonmonoxide on ripening index and respiration.

Reticulocytes suspended in	Atmosphere	cmm oxygen consumed in		Monomolecular constant of ripening rate
		1 hour	6 hours	
Saline	20 % O_2 / 80 % N_2	34	214	0.0148
Saline with 2 % liver extract . .	20 % O_2 / 80 % N_2	34	193	0.0698
do.	20 % O_2 / 80 % CO	25	164	0.0565
do.	10 % O_2 / 90 % CO	18	136	0.0498
do.	5 % O_2 / 95 % CO	14	105	0.0328

Table 8.

Effect of light on respiration and ripening in a carbonmonoxide-oxygen atmosphere.

Reticulocytes suspended in	Atmosphere	Kept in	cmm oxygen consumed in		Monomolecular constant of ripening rate
			1 hour	6 hours	
Saline with 2 % liver extract	93 % N_2 / 7 % O_2	light	43	156	0.0682
do.	do.	darkness	52	149	0.0691
do.	93 % CO / 7 % O_2	light	64	169	0.0691
do.	do.	darkness	11	58	0.0297

The vessels kept in darkness were carefully wrapped in two layers of tinfoil so that no light could reach the content of the vessels.

Discussion.

The results obtained are apparently rather confusing. They can be summarized thus:

1) The oxygen consumption during 6 hours is reduced to a third or less without a corresponding decline in the ripening rate,

2) The addition of a drug in concentrations altering the respiration rate as little as a few per cent gives a similar alteration in the ripening rate,

3) The same is seen when the oxygen pressure is varied,

4) The ripening velocity can be augmented several times, while the oxygen consumption of the cells is decreased rather than increased.

Items 2 and 3 can only be interpreted to mean that the ripening processes in some way or other are linked to the oxygen metabolism of the cell. It may be that the reticular substance acts as a hydrogen donator and its disappearance is simply due to an oxygenation but it may just as well be possible that the ripening process which is observed as a disappearance of the reticular substances, is due to a chemical reaction with an oxygenation product of some other substance found in the reticulocyte, in such a way that one or other step in the oxygenation of this unknown substance reacts with the reticular substance and makes it disappear. It is impossible to decide which of these two explanations is to be preferred as the chemical nature of the reticular substance is not even known yet, but something of this kind must be the case or else the close connection between the oxygen consumption and the ripening rate could not have been observed. Especially the experiments with inhibition of carbon monoxide in darkness and light seem very convincing to the authors.

Even the anaerobic metabolic processes seem to be able to induce some ripening since this is seen to proceed slowly even under completely anaerobic conditions. Experiments to study the ripening when all metabolic processes were excluded by adding monoiodo acetate to blood corpuscles kept under nitrogen did not succeed, as rapid hemolysis occurred.

On the other hand, items 1 and 4 can only be explained if the oxygenation processes linked to the ripening processes form a comparatively small part of the total oxygenation processes in the blood cells. Our experiments show in fact that at least a part of the oxygen uptake has nothing to do with reticulocytes, as some of the non reticulated erythrocytes must have a considerable respiration. This fact, which has not been made clear before, appears from a series of experiments of which we give an example:

As the reticulocytes have a lower specific gravity than the non reticulated cells, it is possible to separate the reticulocytes in a blood sample by means of fractionated centrifugation.

About 40 ml of rabbit's blood with about 100 p. m. reticulocytes was centrifuged at a slow rate (about 1000 r. p. m.). The upper fourth of the blood cells was suspended in saline and centrifuged again in the same manner. If necessary, the upper part from the second centrifugation was separated once more, suspended in saline and centrifuged. The blood cells in the upper layers were then mostly reticulocytes. In the same manner we can isolate non-reticulated cells when the bottom third of the blood-cell layer is centrifuged several times, always taking the bottom third for the next centrifugation. The blood cells were suspended in saline and 3 cc of the suspension were placed in the Warburg vessel and their oxygen uptake measured. The results obtained are given in Table 9.

Table 9.

Total No. of red cells in the vessel	‰ Reticulocytes	Oxygen uptake in cmm in		
		10 min.	30 min.	60 min.
2.29×10^{10}	7	5	17	35
2.32×10^{10}	778	79	178	246

It is seen that although the second sample contains more than a hundred times the reticulocytes of the first sample, the oxygen requirement is only 10—20 times as great. Thus it must be concluded that the non reticulated cells take part in the respiration of the erythrocytes. We have tried to determine how great this part is but find very varying figures. In the experiment of Table 9 the oxygen uptake in the first minute is graphically determined as 0.6 cmm and 10.0 cmm. This gives an oxygen uptake of the reticulocytes about 17 times that of the same number of non reticulated cells. The rest of the experiments showed figures varying from ∞ to 1.8 but with most values lying between 15—30. This variation is certainly due to the fact that not all the non-reticulated cells can respire and the respiring and not respiring blood cells have been fractionated through the centrifugation. When 200—100 p. m. reticulocytes are present and the non-reticulated blood cells have as much as an average respiration of 1/10 of that of the same number of reticulocytes, the respiration of the latter will be three-fourths of the total respiration of the blood sample, i. e. such a large fraction that isolated variations in the oxygen uptake

of the reticulocytes would be registered. This means that the respiration of the non reticulated cells is not sufficient to explain the respiration found not linked to the ripening of the reticulocytes. Therefore one must conclude that even a large part of the respiration processes within the reticulocytes is not linked to the ripening processes.

The oxygenation of the donators, the metabolism of which is linked to the reticulocyte ripening, must apparently have the same mechanism as the oxygenation of the donators having no relation to the ripening processes, as the influence of drugs, reduced oxygen pressure etc. is the same on both processes. The latter donators, however, are used up faster than the former with the result that the rate of oxygen uptake measured after some hours' incubation may be diminished in a considerable degree without a corresponding decrease in ripening rate.

There may possibly be other explanations of the results obtained in the experiments but those mentioned here seem to the authors to be the most likely.

Summary.

We have investigated the relation between the ripening rate of the reticulocytes and the respiration of the blood cells.

1) The ripening rate can be increased several times by means of ripening substances without any increase in the oxygen uptake of the cells.

2) After some hours' respiration the rate of oxygen uptake is considerably decreased, while the rate of the ripening is unaltered.

3) Reduced oxygen pressure reduces both the oxygen uptake and the ripening rate.

4) Drugs with influence on the respiration have a similar influence on the ripening rate. Narcotics, malonate, cyanides and carbon monoxide give a decreased oxygen uptake and a decreased ripening rate, while the addition of fumarate and oxalacetate increase both.

5) The oxygen uptake of the blood cells can be arrested for a few minutes by the addition of oxalacetate.

6) The non-reticulated red blood cells have some respiration too.

7) The results seem to indicate that the ripening of the reti-

culocytes is linked to a part of the respiratory processes of the cells, but the greter part of the respiratory processes has nothing, to do with the development of the unripe blood cell.

References.

ANNAU, E., I. BANGA, B. GÖZSY, ST. HUSZAK, K. LAKI, B. STRAUB and A. SZENT-GYÖRGYI, Hoppe-Seyl. Z. 1935. *236*. 1.
BANGA, J., Hoppe-Seyl. Z. 1936; *244*, 130.
JACOBSEN, ERIK and C. M. PLUM, Acta Physiol. Scand. 1942, *4*, 272, 278.
JACOBSEN, ERIK and C. M. PLUM, Ibidem 1943, *5*, 1.
PLUM, C. M., Ibidem 1942 a, *4*, 259.
PLUM, C. M., Acta Med. Scand. 1942 b, *112*, 151.
PLUM, C. M., Acta Physiol. Scand. 1943, *5*, 165, 175.
WARBURG, O., Ergebn. Physiol. 1914, *14*, 253.
WRIGHT, G. P., J. gen. Physiol. 1930, *14*, 179, 201.

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Physiological Applications of Television Technique: A New Way of Recording Bio-Electrical Phenomena.

By

ERNST BÁRÁNY.

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The usual technique of recording the electrical activity on a surface by placing one or at most a few electrodes on several points simultaneously or in succession is admirably suited for studying the *temporal* course of the potential at every single point but is extremely cumbersome if the object of the study is the exact *spatial* course of the electrical events, for instance the spread of activity on the surface of the heart or the brain. In order to study such problems it would obviously be an advantage if one could visualize and record the electrical activity on the surface in question in the form of images in correct geometrical proportions where the distribution of light and darkness corresponded to the distribution of electrical events. Recording would then be done by cinematography.

It will be shown below that one step in this direction ought to be possible with the aid of modern television technique. The nucleus of the proposed method is a tube which has some features in common with one of the outstanding pick-up tubes of present-day television, the Zworykin Iconoscope. The principles of television with the Iconoscope will therefore be outlined very briefly as an introduction.

In an evacuated glass vessel there is mounted a metal plate covered on one side with a very thin insulating layer. The free side of this layer is given photoelectrical properties by a special

process. When a light image is projected on this pick-up plate each point of the photoelectric layer will lose electrons at a rate proportional to the brightness of the image point falling on it. There will thus develop an electrical *charge image* on the plate. This charge image can be transformed into a television — "video" — signal, which can be used by a television receiver to reproduce the primary light image.

The transformation of the charge image into a video signal is performed by a fine cathode ray pencil of suitable electron velocity (secondary emission factor > 1) which is moved over the charge image in a periodically repeated path that covers the image area. The cathode ray pencil removes the photoelectrically accumulated charge at point after point, the released charge, the video current, is converted into a voltage, amplified and fed into the television receiver, where it is converted into a light image again. In radio television, the video signal is transmitted by radio but this is of no interest here. Thus, the chain of transformations is: primary light image — charge image — point after point dissection into video signal — point after point synthesis into secondary light image.

Now, the basic fact behind the proposed method for visualizing and recording bioelectric phenomena is, that the mechanism of conversion of the charge image into a video signal and then into a light image has nothing to do with the special photoelectric properties of the pick-up tube plate. Thus, *any charge image could be transformed into a light image* by the same arrangement.

There should be no great difficulty in converting the bioelectrical events on a surface into a usable charge image. One possibility is the following.

A very thin insulating sheet is placed on the surface of the organ to be studied. Then, each elemental area of the sheet will act as a small condenser and on the free surface of the sheet there will be a charge image changing with the potential changes at the organ surface.

If, now, the thin insulating sheet were a window in an evacuated vessel, its inner surface could be scanned by a cathode ray pencil exactly as the pick-up plate of the Iconoscope, and a video signal corresponding to the bioelectrical events outside the window would be obtained. Obviously, the charge released from each spot of the window by each passage of the cathode ray pencil would correspond to the *change of potential* beneath that

spot since the last ray passage. Thus, the secondary light image will be an image of the *rates of change of potential* on the surface. A travelling action potential spike on a nerve fibre would thus be represented as a travelling pair of spots, one dark and one light, corresponding to the rising and falling part of the spike.

It is self-evident that the magnitude of the light picture bears no relation to that of the window. Thus, enlarged pictures of the electrical activity on small areas of biological objects might be obtained. Depending on the requirements of the study, the spatial and temporal resolution of the resulting picture will be limited by either the thickness of the window or the thickness of the scanning beam or the band width of the amplifiers. A certain systematic error will be caused by the fact that the potentials of the different spots are recorded at slightly different times. This error can be minimized by increasing the rate of travel of the cathode ray pencil. It is, of course, quite possible that the rates and paths of travel of the ray pencil used in ordinary television are not optimal for recording bioelectrical phenomena. This is not, however, the place to go into details either of apparatus or of the problems that might be approached by the outlined arrangement. At present, only laboratories with quite unusual resources could possibly develop the idea into a working reality. The author does not expect to be able to take part in this work. The aim of the present note is therefore only to point out some of the inherent possibilities of the new technique of television for physiological purposes in the hope that somebody might find the idea worth trying.

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Experimental Studies on Kidney Function during Sulphate Diuresis.

3. Investigations on the Tubular Function of Rabbit Kidneys during Infusion of a Hypertonic Sulphate-solution.[1]

By

PER SCHOU.

Received 4 January 1944.

In a previous paper (SCHOU, 1943) an account was given of the excessive increase in diuresis which can be produced by intravenous infusion of a 20 % Na_2SO_4 solution into rabbits. By this means the diuresis is increased from about 0.1 ml per minute to about 15 ml per minute.

An analysis of how, according to the filtration-reabsorption theory such a diuresis, increased about 150 times, must be accounted for by changes in the glomerular and the tubular function respectively showed that — starting from the creatinine clearance as a measure of the filtration in the glomeruli — partly an increase in the ultrafiltration to two or three times as much as before and partly a very intense inhibition of the reabsorption of water in the tubules must be assumed, so that under these circumstances the urine is only very slightly concentrated.

In another paper (SCHOU, 1944) the pressure and transfusion conditions in the glomeruli were investigated during sulphate diuresis, and it was shown that the physical conditions for the computed increase in the glomerular filtration are probably present.

[1] The work has been supported by a grant from the P. CARL PETERSEN Foundation, Copenhagen.

The object of the present work is to investigate the tubular function during profuse sulphate diuresis. For that purpose the excretion of the various substances occurring in the plasma has been determined, thus of creatinine, the infused sulphate, urea, chlorine, and glucose.

Method.

The present investigations formed a link in the 25 experiments mentioned in a previous paper (SCHOU, 1943).

For the method see the latter. The blood samples taken in the middle of the clearance periods were sufficiently large for the necessary analyses to be made.

Creatinine determination: see the above-mentioned paper.

The *sulphate* in the plasma and the urine was determined by precipitation with benzidine in acetone and subsequent titration with NaOH a. m. COPE (1931) after precipitation of the proteins with trichloroacetic acid.

The *urea* analyses were made on the principle of the urease method by a microdiffusion technique devised by CONWAY and BYRNE (1933).

The *chlorides* in the plasma and the urine were determined a. m. VOLHARD with the technique described by KEYS (1937).

In the *glucose analyses* the method of precipitation of the proteins by means of ferrisulphate and barium carbonate devised by STEINER, URBAN and WEST (1932) and the copper titration method of SHAFFER and SOMOGYI (1937) were employed. The creatinine content had to be known beforehand as a correction had to be made owing to the reducing power of this substance on copper sulphate.

On the basis of these analyses of the urine and plasma, calculations were made for the various substances in order to find:

$$\text{X-C.index} = \frac{\text{mg\% X in urine}}{\text{mg\% X in plasma}}$$

$$\text{X-clearance} = \text{X-C.index} \cdot \text{the diuresis}$$

$$\text{X-Excr.\%} = \frac{\text{mg X excreted}}{\text{mg X filtered}} \cdot 100 = \frac{\text{X-clerance}}{\text{creatinine clearance}} \cdot 100$$

where X stands for the substance analysed.

Results.

In Tables I and II the results of these calculations are given together for the 6 experiments which were used as examples in the preceding paper (SCHOU, 1943). The figures for the diuresis and for the creatinine clearance (Table I) as well as for the

concentration index (= C.index), (Table II), will be found again here.

Table I with the following Table II show the results of 6 of the sulphate diuresis experiments.

For each of the experiments are given the figures for a normal period (N) and 3 sulphate diuresis periods (S_1, S_2, and S_3).

The ultrafiltration in the glomeruli is calculated as creatinine-clearance.

The figures for the diuresis are those directly found, expressed in ml per minute.

Further the figures for the concentration of the various substances in the serum are given, as well as the amounts of the substances in question which are excreted in the urine.

Table I.

Experiment	Period	Creatinine-Clearance ml per minute = Filtration	Diuresis ml per minute	Concentration in serum mg per 100 ml = mg%				mg excreted per minute in urine			
				Sulphate	Urea	Chlorine	Glucose	Sulphate	Urea	Chlorine	Glucose
15	N	11.9	0.1	22	39.6	385	463	0.65	2.18	0.1	—
	S_1	26.3	13.0	392	38.6	341	413	88.0	8.89	36.0	60.0
	S_2	24.0	12.4	499	37.5	330	365	105.0	7.52	30.9	48.8
	S_3	16.3	8.5	593	37.2	318	357	90.7	6.13	15.7	25.7
16	N	7.6	0.1	18	75.8	392	586	0.33	2.69	0.2	—
	S_1	16.3	7.8	333	70.6	366	465	51.8	12.32	23.7	49.3
	S_2	17.2	11.9	485	65.5	337	413	87.2	11.65	34.2	58.4
	S_3	10.9	7.8	500	69.1	325	385	57.7	7.47	18.8	34.6
17	N	9.0	0.07	15	41.0	375	361	0.02	0.98	0.2	—
	S_1	34.8	8.3	188	40.0	338	367	30.4	9.90	23.8	28.8
	S_2	29.5	15.4	291	39.0	325	347	65.1	9.30	48.3	53.3
	S_3	18.1	12.4	327	38.3	312	321	67.8	6.87	34.1	40.9
18	N	12.0	0.05	16	25.5	373	396	0.2	0.79	0.04	—
	S_1	22.5	11.3	303	25.0	352	358	73.1	5.90	27.4	43.8
	S_2	24.5	16.3	438	25.0	319	344	110.5	6.43	39.8	53.0
	S_3	15.9	8.0	521	24.0	302	348	84.5	3.84	12.8	19.0
19	N	13.6	0.05	—	38.8	367	451	—	0.35	0.1	—
	S_1	21.6	7.5	288	38.6	344	427	55.1	7.30	17.3	48.2
	S_2	23.6	15.3	425	35.3	333	492	110.2	9.21	36.8	86.6
	S_3	16.7	11.0	588	34.4	314	517	104.5	5.92	24.8	63.4
20	N	10.8	0.08	15	24.0	389	415	0.15	0.82	0.2	—
	S_1	20.6	11.6	322	24.8	346	759	73.3	5.62	33.7	105.2
	S_2	22.2	15.5	529	23.9	312	831	117.0	5.55	43.3	145.2
	S_3	14.0	8.3	588	24.1	307	781	80.2	3.22	16.8	88.0

Table II shows (from the same 6 experiments as in Table I):
The degree of concentration in the urine of the various substances examined = C. index (the creatinine-C. index expresses the degree of concentration for the urine itself).
The Excr.% as an expression of that part of the ultrafiltrated amounts of the substances which is excreted with the urine.

Table II.

Experiment	Period	C. index					Excr.%				
		Creatinine (= "urine")	Sulphate	Urea	Chlorine	Glucose	Water	Sulphate	Urea	Chlorine	Glucose
15	N	125	30.5	57.8	0.27	—	0.8	24	46	0.2	—
	S_1	2.02	1.73	1.77	0.81	1.12	49	86	88	40	55
	S_2	1.93	1.70	1.62	0.75	1.08	52	88	84	39	56
	S_3	1.91	1.80	1.94	0.58	0.85	52	94	101	30	44
16	N	54.1	13.0	25.3	0.30	—	1.9	24	47	0.7	—
	S_1	2.10	2.01	2.25	0.83	1.37	48	96	107	40	65
	S_2	1.45	1.51	1.50	0.86	1.19	69	105	103	59	82
	S_3	1.41	1.49	1.39	0.74	1.16	71	106	99	53	82
17	N	138	2.07	37.0	0.60	—	0.7	2	27	0.6	—
	S_1	4.22	1.96	3.00	0.85	0.95	24	47	71	20	23
	S_2	1.92	1.45	1.55	0.97	1.00	52	76	81	50	52
	S_3	1.46	1.67	1.44	0.82	1.03	68	114	98	61	70
18	N	250	27.0	65	0.20	—	0.4	11	26	0.1	—
	S_1	2.00	2.14	2.10	0.69	1.09	50	107	105	35	54
	S_2	1.51	1.55	1.58	0.77	0.95	66	103	105	51	64
	S_3	1.98	2.03	2.00	0.53	0.68	50	102	101	27	35
19	N	303	—	19.9	0.43	—	0.3	—	6	0.1	—
	S_1	2.88	2.55	2.52	0.67	1.50	35	89	88	23	—
	S_2	1.55	1.70	1.71	0.72	1.15	65	110	110	47	75
	S_3	1.52	1.62	1.56	0.72	1.12	66	106	103	47	74
20	N	138	10.5	43.8	0.50	—	0.7	9	32	0.5	—
	S_1	1.77	1.96	1.95	0.84	1.19	56	110	110	47	67
	S_2	1.43	1.43	1.50	0.87	1.13	70	100	104	61	79
	S_3	1.70	1.65	1.62	0.67	1.37	59	97	95	39	80

The following facts emerge:

1) The very great increase in the diuresis from the c. 0.05—0.1 ml per minute of the normal periods to the 7—16 ml per minute of the sulphate diuresis periods.

2) The increase of the glomerular filtration to about 2—3 times the normal during the sulphate diuresis and

3) the immense fall in the creatinine-C.index (= the concentration of the urine) from the normal values of 50—300 to figures ranging round 2.0—1.5 at the height of the sulphate diuresis.

These last figures must accord with the fact that $^1/_2$—$^2/_3$ of the glomerular filtrate under these circumstances pass down through the tubular canals and then pass out as urine (i. e. the water-Excr. % = 50—66) while correspondingly only the second half to a third part can be reabsorbed.

Such extremely low figures for the renal concentration action must of course lie far below what will occur in the kidney except in experimental work, and in this connection it is worth noting that in no case within these experiments have figures *lower* than 1 been found for the "urine"-C.index.

If this had been the case it would not have been possible to explain the renal function during sulphate diuresis by the filtration-reabsorption theory, since this would mean that the glomerular filtrate far from being concentrated in the tubules would on the contrary have been diluted on its way through these and the excretion of fluid necessitated by this is not considered in the theory.

The sulphate excretion. Table I shows that the sulphate content of the serum *in the normal periods* was found to be about 15—20 mg% SO_4 and that only small amounts of sulphate, less than 1 mg per minute are excreted with the urine.

The figures for the C.index show that the sulphates are concentrated in somewhat varying degree, but not so much by far as the ultrafiltrated fluid. The experiments here reported exhibit C.indices between 2 and 30 for sulphate, and as previously stated, between 54 and 303 for the glomerular filtrate.

The corresponding figures for the sulphate-Excr.% (Table II) lie between 2 and 24. Since it is certain that sulphate passes freely through the glomerular membrane this must probably mean that under normal circumstances a considerable reabsorption of sulphate takes place in the tubules, as has indeed been conjectured by several authors (Cope 1932, Hayman and Johnston 1932, Bjering and Øllgaard 1939).

In *the sulphate diuresis periods* the serum sulphate is found to come very near to about 600 mg%, and a considerable excretion

of sulphate now takes place in the urine, up to over 100 mg per minute.

The excretion conditions, too, are quite different from those of the normal periods.

The figures for the sulphate-C.index (Table II) here come quite near to the simultaneous figures for the "urine"-C.index, and

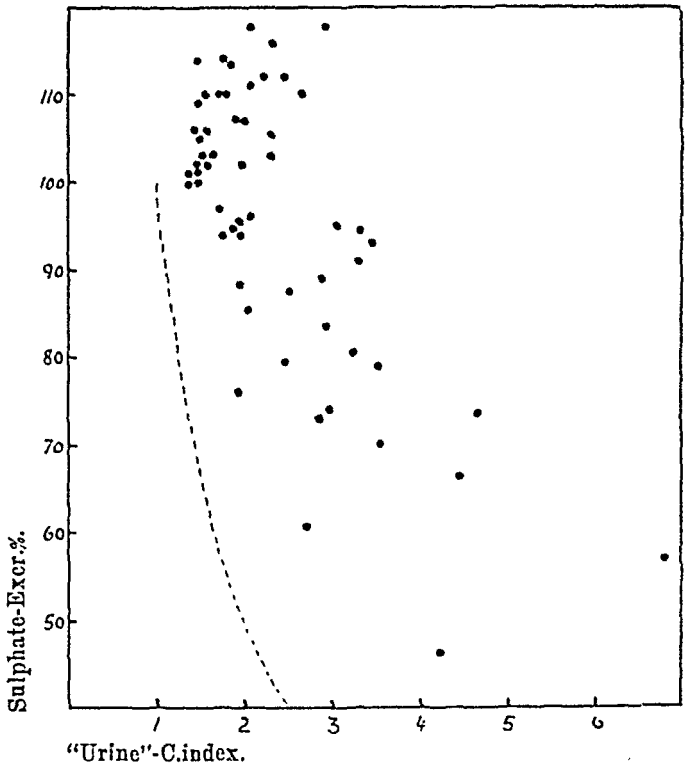

Fig. 1 shows the relation between the degree concentration of the urine = the "urine"-C.index and the sulphate-Excr.% in 56 sulphate diuresis periods from 17 experiments.

The stippled line is the curve constructed as per the definition of the relation between the "urine"-C. index and the water-Excr.%.

the corresponding figures for the sulphate-Excr.% must thus group themselves round 100, that is to say, there is agreement between the sulphate and the creatinine clearance.

Fig. 1 shows the relation between the sulphate-Excr.% and the "urine"-C.index. In the diagram has been traced what might be called the "water excretion curve", i. e. the curve constructed as per the definition of the relation between the water-Excr.% and the "urine"-C.index. According to the definition of these concepts this curve must be an equilateral hyperbola, since the

Excr.% $= \frac{1}{\text{C.index}} \cdot 100$. At a urine concentration index of 2, 50 % of the ultrafiltrate must be reabsorbed.

Fig. 1 shows in the first place the general tendency that the less the concentration of the urine, the greater is the part of the filtrated sulphate that is excreted, that is to say, the less will be reabsorbed, until at the lowest values for the concentration index of the urine, a complete cessation of the reabsorption of sulphate must be assumed.

In the second place, the diagram shows that all the points lie *above* the "water excretion curve". The sulphate-Excr.% then is always greater than the simultaneous ordinary water-Excr.%. This expresses the fact that the concentration of sulphate in the urine was in all cases found to be higher than the simultaneous concentration of the plasma. Where a reabsorption of sulphate takes place in the tubules this is never greater than what might be explained by a passive diffusion process.

It will be noted that the diagram presents values for the sulphate-Excr.% right up to 115. Theoretically the occurrence of figures above 100 for this factor should be interpreted as an expression of an *excretion* of the substance in question in the tubules, but it should here be kept in mind that the sulphate-Excr.% factor is the result of calculations on the basis of 4 analyses in all and a measurement of the diuresis, the errors of which may sum up, and that the calculations, especially concerning the *sulphate* excretion, in these experiments must become somewhat uncertain owing to a greatly fluctuating sulphate content in the plasma as a result of the rapid infusion. Thus it will probably be most prudent to interpret these too high points as a deviation in the above-mentioned grouping round 100 resulting from an experimental error.

The excretion of urea. The content of urea in the serum (Table I) varies in the individual experiments between 25 and 75 mg per 100 ml, but is otherwise remarkably constant in the individual experiments irrespectively of the great changes in the composition and concentration of the plasma caused by the sulphate infusion.

The excretion of urea in the urine rises during the sulphate diuresis from about 0.5—2.5 mg per minute up to 11—12 mg per minute.

In other respects the excretion of the urea presents essentially the same picture as that of the sulphates.

REHBERG (1926) regards urea as a no-threshold substance which is filtered with the plasma into the glomeruli and then passively diffused back into the blood through the tubular wall to the extent permitted by the degree of concentration of the urine.

This is in fact found to be the case in the *normal periods* of these experiments. It is found (Table II) that the urea is considerably less concentrated than the urine itself. In all the experiments

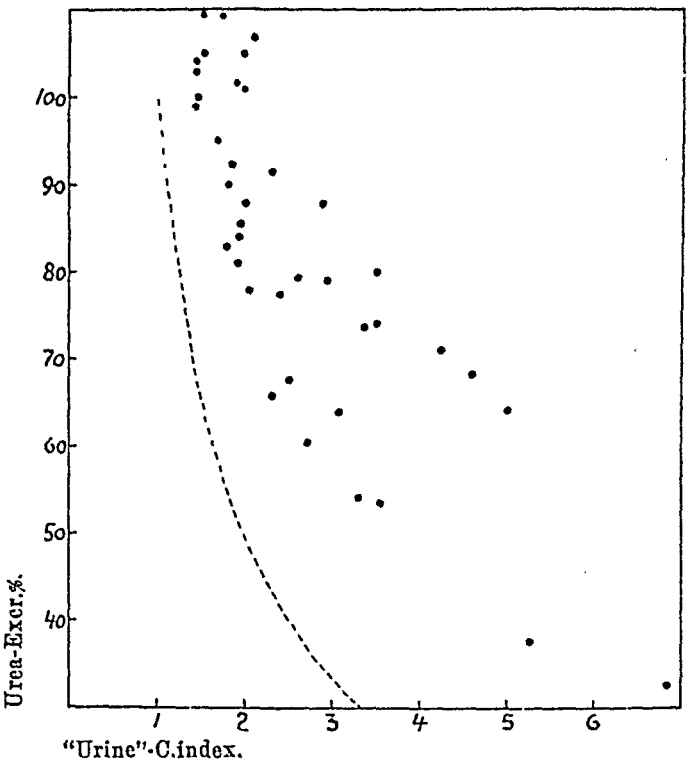

Fig. 2 shows the relation between the "urine"-C.index and the urea-Excr.% in 40 sulphate diuresis periods from 14 experiments.

As to the stippled curve - - - - - - - - see the text to Fig. 1.

(apart from a single case in experiment 19 of this table) the urea-Excr.% ranges round 20—50.

In *the sulphate diuresis periods*, on the other hand, it is found that the urea has the same degree of concentration as the urine, that is to say, they have virtually identical concentration indices. So that just as in the case of sulphate, so also in the case of urea the figures for the Excr.%, at any rate at the height of the diuresis, range round 100.

Fig. 2 shows the relation between the degree of concentration

of the urine and the Excr.% of the urea. The plotted curve (. . .) as stated above as far as the sulphate is concerned shows the relation between the "urine"-C.index and the water-Excr.%.

The diagram is exactly similar to the corresponding one for sulphate. The urea-Excr.% shows a distinct tendency to rise gradually as the degree of concentration of the urine decreases, until at concentration indices of 2—1.5 a total excretion of the ultrafiltrated urea is found.

As to the values for the urea-Excr.% exceeding 100, here the same considerations must apply as were stated above for the sulphate.

All the points in the diagram lie above the "water-excretion curve", expressing that in no case was less urea found in the urine than in the plasma.

These two observations show that the principle of the excretion of urea under these circumstances is the same as that demonstrated by REHBERG under normal circumstances, that is to say, that the back diffusion of urea is proportional to the degree of concentration of the urine.

This has indeed been confirmed by SHANNON (1938), for increased diuresis too.

It will be noted that, as was the case with the filtrated fluid, no values for the C.index less than 1 have been found either for sulphate or for urea, a fact which highly favours the supposition that the *principle* of the mode of excretion for these substances is the usual one in the kidney only here adapted to the unusual working conditions.

The chlorine excretion. The serum concentration in the *normal periods* is seen in Table I to be somewhat variable in the individual experiments — between 370 and 390 mg per ml, probably as a result of the intravenous infusion of varying amounts of 0.9 % NaCl which was necessary to prevent coagulation in the cannula.

In spite of the rather high content of chlorine in the plasma the excretion of chlorine was but slight, 0.2 mg per minute or less. Hence the animals must have been rather poor in chlorine.

After the *sulphate infusion* was begun, a distinct reduction in the plasma of the chlorine concentration to about 300—320 mg % was constantly seen in all the experiments.

This might perhaps be explained as a simple consequence of the dilution of the serum caused by the infusion, but at the same time the figures for the chlorine content of the urine show a very

considerable increase in the chlorine excretion, to about 40 mg per minute.

Similar observations on the ability of the sulphate ion to "replace" the chlorine ion in the plasma have been made by MØLLER (1926) and by AMBERSON, NASH, MULDER, and BINNS (1938) who showed in experiments on cats that in this way the chlorine in the plasma may be reduced by as much as 25 %.

According to REHBERG (1926) the mode of excretion of the chlorine is dependent on the concentration of chlorine in the plasma, in such a way that at low concentrations of chlorine in the plasma — below the threshold about 370 mg % — it is supposed to be actively reabsorbed, whereas at a higher figure an excretion of this substance on a line with the no-threshold substances is assumed.

The mode of excretion of the chlorine in the *normal periods* of these experiments is indicated by the few examples in Table I. It is seen that here, in rabbits, the excretion of chlorine follows the same lines as REHBERG found for humans. The chlorine content of the urine is considerably less than that of the plasma, so that the chlorine-C.index lies considerably below 1. The chlorine-Excr.% shows that when the chlorides of the plasma, as was the case in these experiments, range round the normal 370 mg %, less than 1 % of the chlorine which is filtered enters the urine, while the rest passes back into the blood through the tubular wall.

During *the sulphate diuresis*, values for the chlorine-C.index are found which are somewhat higher than in the normal periods, though still less than 1; this expresses the fact that the chlorine concentration in the urine approaches the concentration in the plasma.

This relatively slight change in the chlorine-C.index will, however, when viewed in connection with the enormous excretion of fluid, signify a very considerable increase in the excreted amount of chlorine per minute — as already mentioned above.

How the increased excretion of chlorine is brought about is seen from the chlorine-Excr.% which, from the above-mentioned values below 1, rises to between 20 and 60. This is apparently in conflict with what REHBERG found for the chlorine excretion in man, viz. that at low values of chlorine in the plasma the chlorine-Excr.% is practically independent of the C.index of the urine, lying at about 1—2 %. But this fact too must be viewed in the

light of what was said above about the excretion of fluid. Under these circumstances so little fluid is reabsorbed that the kidneys can only to a very limited extent carry the chlorine back to the blood, and in this way the excretion of chlorine must reach a much higher level.

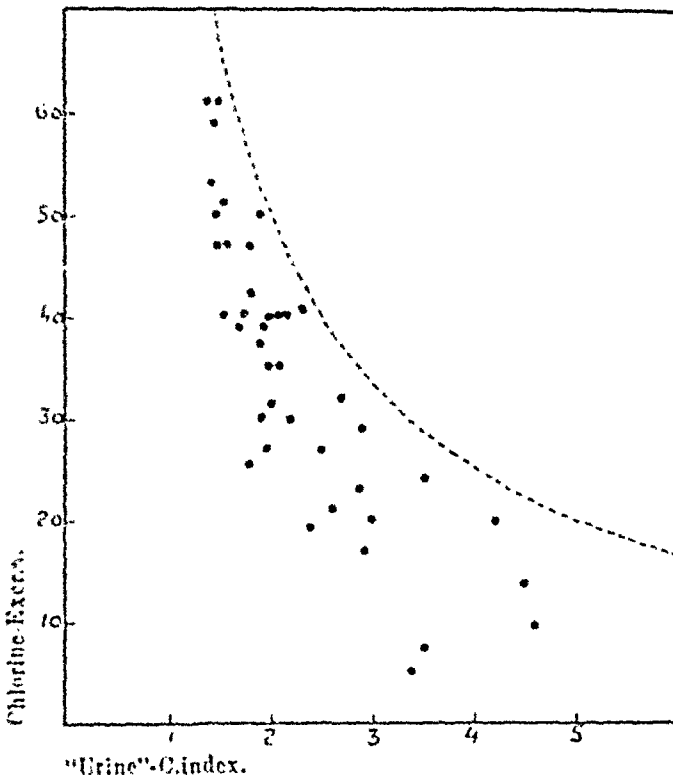

Fig. 3 shows the relation between the "urine"-C.index and the chlorine-Excr.% in 14 sulphate diuresis periods from 14 experiments.
As to the stippled curve - - - - - - - - see the text to Fig. 1.

As will appear from the diagram Fig. 3, the less the urine is concentrated, that is to say, the less of the glomerular filtrate is reabsorbed, the less of the filtered chlorine will pass back through the tubules, showing that the chlorine-Excr.% rises with the falling concentration index.

As far as chlorine is concerned, all the points lie *below* the above-mentioned "water excretion curve". If for instance we consider period 1 in experiment 18 we shall find (Table II) a water-Excr.% of 50, that is to say, a concentration index of 2. At the same time the chlorine-Excr.% is 35, and thus in Fig. 3 will lie lower than the "water excretion curve".

This means, then, that 50 % of the fluid filtrated is reabsorbed. This reabsorbed amount of fluid is to contain 65 % of the chlorine that has been filtered. Hence the chlorine content of the fluid which passes back through the tubular wall must be 30 % higher than in the plasma.

In all the experiments on the chlorine content in the reabsorbed fluid the calculations have in fact shown a higher chlorine content in this than in the serum within the same periods (figures not given).

As previously stated, the values for the chlorine content of the serum were below 370 mg %.

This circumstance must be regarded as expressing the fact that even if, gradually as the concentration index falls, a steadily increasing amount of the filtrated chlorine is excreted, still the active chlorine reabsorption will not cease entirely at any time.

The excretion of glucose. Table I shows that in these experiments the content of glucose in the plasma increases in a considerable degree to about 300—500 mg %, that is to say, to far above the threshold value. In experiments 15—19 incl. this increase must be due to the administration of uretane, whereas the further rise, in experiment 20, to more than 800 mg % is due to the administration to this animal of 4 gr of glucose for the purpose of examining the excretion of glucose at corresponding high values for glucose in the plasma.

Glucose was excreted in the urine in all periods but the glucose excretion in the normal periods was not examined since the high content of creatinine in the concentrated urine of these periods rendered glucose analysis impossible.

The figures for the glucose concentration in the urine are not given in the tables but they were found to lie around the simultaneous glucose concentrations in the plasma, though in most of the periods a trifle higher.

Thus the glucose-C.index will range around 1.0 (Table II), and in fig. 4 it is seen that the points which in the individual periods of diuresis show the relation between the glucose-Excr.% and the "urine"-C.index similarly lie near the "water excretion curve", in by far the greater part of the cases a little *above* it, only in some few of the 30 cases below the curve.

Theoretically, then, this would mean that only in these few cases have the tubules been able to maintain a glucose reabsorption in the same active sense as for chlorine, while in most of

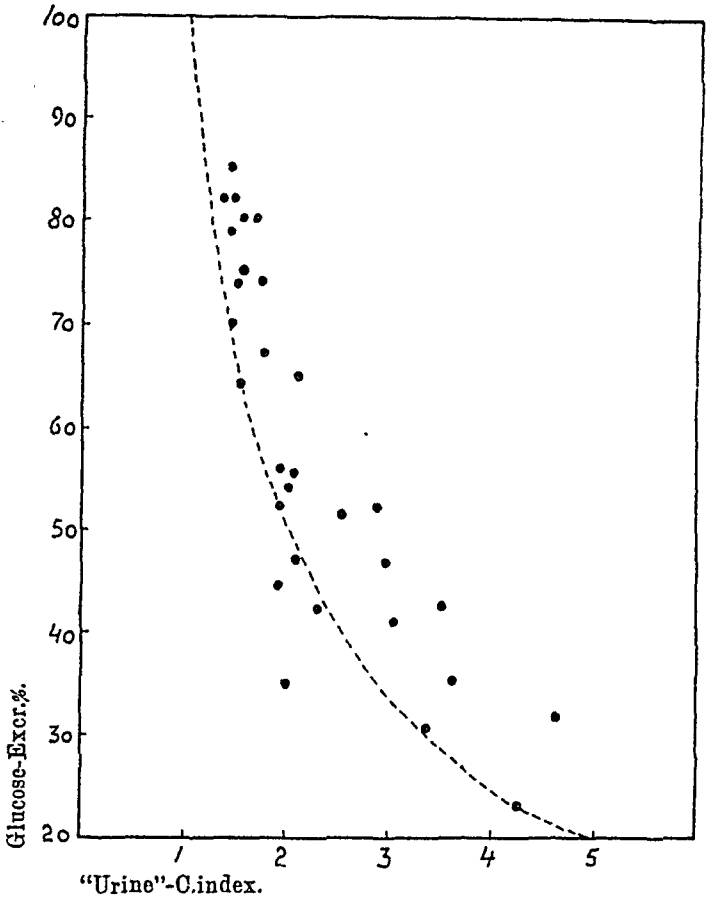

Fig. 4 shows the relation between the "urine"-C.index and the glucose-Excr.% in 30 sulphate diuresis periods from 10 experiments.
As to the stippled curve -------- see the text to Fig. 1.

the periods it should be possible to explain the reabsorption mechanism as a simple diffusion process.

But, on the other hand, the diagram shows that the kidneys at a concentration index of 2.0 can still transfer 40—60 % of the glucose back to the blood, which is perhaps most easily interpreted as a result of an active process.

When the degree of concentration of the urine falls below 2.0 the reabsorption decreases very quickly, that is to say, the glucose-Excr.% tends to approach 100 without quite reaching this figure.

Thus it proves that the same inverse proportionality which was observed in greater or less degree for the other substances also asserts itself here as an expression of the fact that the less the urine is concentrated the less of the filtrated glucose passes back through the tubular wall.

Discussion.

The concentration in the serum of the various substances surveyed in Table I shows that it is in the main the fluctuations in the chlorine ion and sulphate ion content which determine the changes in the content of electrolyte in the plasma from the normal periods to the sulphate diuresis periods.

If the concentrations stated are converted into milliequivalents per liter of serum it turns out that the rise in the sulphate concentration is far greater than — 5 times as great as — the reduction in the chlorine concentration.

The sum of Cl^- and SO_5^{--} ions in the experimental periods constitutes about 110 milliequivalents per liter, while the figure after the sulphate infusion may lie at about double that amount (figures not given).

Thus there is a very considerable increase in the content of electrolytes in the plasma.

Though it is not possible to make any definite statement as to how the excretion of this enormous surplus of electrolytes is regulated, it may still be pointed out that the kidneys excrete the sulphate ions as quickly as it is at all possible according to the filtration-reabsorption theory, that is to say, by an increased filtration and a virtual cessation of the reabsorption of this substance, and further, that it seems that the organism lets the sulphate ions forced upon the plasma replace up to 20 % of the chlorine in the plasma, which can thus also be excreted in the urine in considerable amounts.

In a previous paper (SCHOU, 1943) it was stated that when up to 60—70 % of the glomerular filtrate is excreted as urine during the sulphate diuresis, the urine must approach the ultrafiltrate in composition if the filtration — reabsorption theory is to hold good under these circumstances.

The investigations reported in the present paper have now shown that this is in fact the case, as the concentration of the various substances examined has proved to be reduced and "made uniform" to such a considerable extent that from widely differing values the figures for the concentration indices for these substances approach values ranging round 1.

It is true that under these circumstances the excretion conditions differ widely from what is offered the kidneys under normal physiological conditions; it may be pointed out, however, that

— as was the case for the excretion of fluid (SCHOU, 1943) — so also as far as the substances contained in the tubular fluid are concerned conditions were in no case met with which cannot in principle be explained by the filtration-reabsorption theory.

The experimental results have shown that the characteristic factor in the tubular function sulphate diuresis is a general failure of the reabsorption process.

CUSHNY (1926) studied the mechanism of the sulphate diuresis and advanced the view that the presence of the not easily reabsorbable sulphate ion in the tubules by its osmotic effect had an inhibitory influence on the reabsorption of water.

The experiments reported in the present paper have shown that the decisive factor in the excretion of all the substances examined is precisely the very low degree of concentration of the urine-C.index, caused by the failing reabsorption of *fluid*, possibly with the high concentration of sulphate in the urine as the primary factor.

Though no conclusive explanation can be given of the *quantitative* regulation of the reabsorption process under those circumstances outside the ordinary, it should, however, be possible, on the basis of the experimental results of the present work — and with support from CUSHNY's standpoint — to put forward the following view concerning the tubular function during sulphate diuresis.

After the direct infusion into the bloodstream of large amounts of hypertonic sulphate solution the renal tubules receive through the filtration in the glomeruli an ultrafiltrate with a very high content of sulphate ion. This can only with great difficulty pass back through the tubular wall, so all the way down through the tubules it will prevent the reabsorption of fluid by its osmotic effect.

In this way the conditions for a normal reabsorption of the other substances occurring in the tubular fluid are compromised, for, owing to the failing reabsorption of fluid, they cannot pass back through the tubular wall to the extent that must be presumed to be the case in the usual renal treatment of these substances.

Consequently it must be supposed that the osmotic effect of these substances, in conjunction with the sulphate ion, must in some way or other assert itself in the tubules in the final regulation of the extent of reabsorption, and so of the composition and amount of the urine.

Conclusion.

By experiments reported in previous (SCHOU, 1943, 1944) and in the present paper it is shown that it should be possible to explain the profuse sulphate diuresis as a result of a combination of an increased filtration in the glomeruli and a much reduced reabsorption of fluid, and so of the substances dissolved in the fluid, in the tubules.

The explanation of this very great excretion of fluid will thus be found consistent with the filtration-reabsorption theory on the renal function, and the experiments will therefore — as was the main object of the work — serve to confirm the general validity of that theory even though the excretion of fluid rises to many times that which the kidney must yield under normal circumstances.

Summary.

In continuation of papers previously published by the author on renal function after infusion of a hypertonic sulphate solution into rabbits, the present paper gives an account of the function of the tubules during sulphate diuresis. The excretion of the substances contained in the plasma has been investigated through clearance determinations.

1) *Sulphate.* The experiments show that before the sulphate infusion a considerable reabsorption of sulphate ion must be assumed. During the sulphate diuresis this is reduced until at the height of the diuresis it completely ceases, that is, the sulphate-Excr.% = about 100.

2) *Urea* shows in principle the same conditions.

3) *Chlorine.* The reabsorption of chlorine is considerably reduced, but it is still thought possible to observe an active reabsorption of chlorine in the tubules. The chlorine-Excr.% rises from less than 1 to 20—60 %.

4) *Glucose.* During the sulphate diuresis the glucose Excr.% rises to more than 80 % gradually as the degree of concentration of the urine decreases.

Here — as for the other substances — it holds good that the reabsorption decreases very rapidly when the C.index of the urine falls below 2.

5) The decisive factor in the excretion of all the substances

studied is the low C.index of the urine, resulting from the failing reabsorption of fluid.

All the conditions observed during the sulphate diuresis can be explained by the filtration — reabsorption theory.

References.

Amberson, W. R., Th. B. Nash, A. G. Mulder and D. Binns, Amer. J. Physiol. 1938. *122.* 224.

Bjering, T., and E. Øllgaard, Acta med. Scand. 1932. *102.* 55.

Conway, E. J., and A. Byrne, Biochem. J. 1933. *27. 1.* 419.

Cope, C. L., Ibidem 1931. *25. 4.* 1183.

—, J. Physiol. 1932. *76.* 329.

Cushny, A. R., The secretion of urine. London 1917 and 1926.

Hayman, J. M. Jr, and S. M. Johnston, J. clin. Invest. 1932 *11.* 607

Keys, A., J. biol. Chem. 1937. *119.* 389.

Møller, K. O., Om vand- og saltskiftet under forskellige former af forøget diurese. Diss. Copenhagen 1926.

Rehberg, P. B., Biochem. J. 1926. *20.* 447.

Schou, P., Experimentelle undersögelser over sulfatdiuresen, Diss. Copenhagen 1942.

—, Acta Physiol. Scand. 1943. *6.* 79.

—, Ibidem 1944. *7.*

Shaffer, H. A., and M. Somogyi, J. biol. Chem. 1933. *100.* 695.

Shannon, J. A., Amer. J. Physiol. 1938. *122.* 782.

Steiner, A., F. Urban, and E. S. West, J. Biol. Chem. 1932. *98.* 289.

From the Institute of Pharmacology, University of Copenhagen.

Experimental Studies on Kidney Function during Sulphate Diuresis

4. Investigations on the Kidney Function in Rabbits with Chronic Tubular Nephritis a. m. Frandsen.[1]

By

PER SCHOU.

Received 4 January 1944.

In a number of previous papers (SCHOU 1943, 1944) the author gave an account of the profuse diuresis resulting from intravenous infusion of a hypertonic sulphate solution into rabbits.

Investigations on the mechanism of this diuresis showed that, if it is to be explained on the principle of the CUSHNY-REHBERG filtration-reabsorption theory, the fundamental factors of the enormous excretion of fluid must be partly an increase in the filtration in the renal glomeruli, partly a very considerable reduction of the reabsorption of fluid in the tubules.

As far as the glomerular function is concerned it has been possible to show *directly* that the blood pressure and the renal blood flow are appreciably increased as a result of the sulphate infusion, so that physically an increased filtration should be possible.

With respect to the tubular function the assertion of its partial failure rests on more *indirect* evidence, viz. on calculations based on that theory of kidney function, the validity of which it has, precisely, been the object of these papers to confirm.

Considerations of this kind suggested the possibility of investigating the kidney function during sulphate diuresis on kidneys *whose reabsorption ability had already been compromised* by an injury to the tubules.

[1] This work has been aided by a grant from the P. Carl Petersen Foundation, Copenhagen.

If it could be shown in this way that kidneys whose tubules are not or only in slight degree capable of functioning, will function in the same way as normal kidneys after a sulphate infusion, this would furnish more direct support for the theory of the tubular function during sulphate diuresis.

In his doctor's dissertation (1923) and in later works (1925, a, b, and c) on the same subject FRANDSEN has described a technique by which it is possible to produce experimentally a *chronic tubular nephritis* in rabbits by means of repeated intravenous injections of small doses of potassium bichromate. What would make this form of nephritis particularly well suited for the object of *this* work is that according to FRANDSEN's microscopic examination it leaves the glomeruli virtually intact, at any rate as long as the nephritis has not risen to very high degrees.

Experimental Technique.

Employing FRANDSEN'S technique for producing nephritis, sulphate diuresis experiments were therefore made on three such nephritic rabbits, the technique and method of analysis being exactly the same as were previously indicated by the author (1943). A very brief description of the procedure will therefore suffice here.

As already mentioned, the principle of the experiments is to investigate the excretion of water and various substances dissolved in this during quite short periods after the diuresis has been increased by means of intravenous infusion of a hypertonic sulphate solution.

The two first experiments were based on 4 minute periods, the third experiment on 2 minute periods.

As in the previous experiments, the plasma and urine were analysed for creatinine, sulphate, urea, chlorine, and glucose to determine the degree of concentration in the urine, i. e. the *C.index*, and by means of the figures for the diuresis the values were calculated for the clearance as well as the figures for the *Excr.*% = the percentage of water filtrated in the glomeruli, as also the various substances which after passing through the tubules come out as urine.

The animals used were male rabbits weighing 2.5—3 kg. In the course of four months before the experiments they were injected every 6th day through an ear vein with about 0.8 ml of a 4 % solution of potassium bichromate, 21 times in all, the last chromate injection being given 6 days before the experiments.

The animals were given the usual diet of turnips, oats and hay. Two of the animals kept their weight throughout the period preceding the experiment, the third lost 650 gr. In all three experiments the urine contained albumen on the day of the experiment.

As an example we may give the experimental notes from the third experiment.

Experimental notes

At 13.15 administration of 17.5 ml 25 % uretane solution + water to 100 ml by mouth. The animal is then put in the incubator.
14.20 administration of 1 gr creatinine in 20 ml 0.9 % NaCl solution subcutaneously. Operation in the usual way.
15.31—.55 normal period (N) with quite slow intravenous infusion through the inserted cannula of 20 ml 0.9 % NaCl solution in all.
16.12—.24 infusion of 75 ml 20 % $Na_2SO_4 \cdot 10H_2O$ solution.
16.17—.23 1st, 2nd, and 3rd diuresis period of 2 minutes each (S_1 S_2 and S_3).
16.24 the experiment is interrupted, the animal killed, the kidneys removed, and at once fixed in formol-Zenker's fluid.

The histological picture of the kidneys of the experimental animals.[1]

The kidneys prove to be large, heavy, edematous, somewhat pale, with red cyanotic patches.

In sections the width of the cortical zone is seen to be reduced. The base of the pyramids is uniformly red without the usual striation, and there is a sharp line of demarcation between this zone and the larger collecting tubes at the apices of the pyramids, which have a natural appearance.

Histologically most of the glomeruli are moderately enlarged. Some few of the glomeruli, however, show incipient shrinking. Locally is seen an increase of the connection tissue fibrils in the interstitial tissue. In a few glomeruli there is round cell infiltration and here and there leucocytes appear.

The convoluted tubules present a varied, heterogeneous picture, above all dominated by the enormously dilated lumina. In by far the greater number of the transverse sections of the ducts the dilated cells are for the most part seen to be rubbed off so that of the original epithelial coating only the basal membranes are seen and quite few surviving cells which have become quite flattened.

In numerous ducts there is thus no coating of cells at all, in others the cells are preserved but the seat of considerable degeneration, and in a few of the tubules grouped round a few glomeruli the cells may have an almost quite natural appearance with deeply stained nuclei and protoplasm and well preserved brush seams.

There are all transitions beteeen these stages but the picture is predominantly marked by the quite flattened cells lying like a zyncytium round the dilated lumina.

The farther one gets down into the medullary rays and the pyramid, presumably on a level with the tubuli recti, and the small collecting

[1] I am much indebted to prosector Dr. L. HEERUP for his aid in the analysis of the histological preparations.

tubes, the more one sees a steadily increasing occurrence of hyaline cylinders in the largest dilated lumina, but otherwise the cells in the collecting tubes, especially in the largest ones, gradually as one approaches the tips of the papillae become more and more natural in appearance with distinct cell boundaries and distinct not degenerated nuclei.

Throughout the kidneys is seen a diffusely increased development of connecting tissue, particularly pronounced around the vessels which are otherwise unchanged.

Thus the kidneys present the picture of a pronounced chronic degeneration of by far the greater number of the tubules.

It would seem very little probable that there should be a possibility of any active reabsorption process in the tubules, where the cells have been most injured, but in between there are more well-preserved areas which, judging from the picture, could very well be localised to the course of the individual nephrons which have thus for some reason or other proved refractory to the influence of the chromatic acid.

As to the glomeruli, they are for the most part well preserved though here and there is seen an incipient change in them sufficient to explain the lack of ability, mentioned below, to augment the filtration in the same degree as in healthy animals.

Experimental Results.

The results will appear from Table I in which, omitting numerous intermediate calculations, the computed factors have been presented, which are necessary to estimate the kidney function in the three nephritic animals. The corresponding figures from two of the sulphate diuresis experiments (experiments 18 and 19) on healthy animals mentioned in previous works (SCHOU 1943) are inserted for comparison.

For the *normal periods* (N) before the sulphate infusion the table shows that the *diuresis* is somewhat larger here in the nephritic animals (0.17, 0.51 and 0.43 ml per minute) than in the healthy animals (about 0.05 ml per minute).

The filtration in the glomeruli, on the other hand, is considerably lower in the nephritic animals (1.3, 3.5 and 4.3 ml per minute) than in the healthy animals (12.0 and 13.6 ml per minute).

These facts are expressed in the *urine-C.index* which in these experiments shows much lower values for the nephritic kidneys (7.6, 6.9, and 9.9) than for the sound kidneys (250 and 303).

The corresponding *water excretion* % in the nephritic animals is in these experiments 13, 14.5, and 10.1, whereas this factor under normal circumstances lies below 1 (in the examples given here 0.4 and 0.3).

Table I shows the observed and the calculated factors expressing the excretion of water through the kidneys.

Further, the mode of excretion for sulphate, urea, chlorine, and glucose is expressed through the Excr.% of these substances.

Excr.% = the percentage of the amount of fluid calculated to be filtered in the glomeruli, as also the amount of the substances which passes out with the urine.

For each experiment the table comprises a normal period (N) prior to the sulphate infusion and three sulphate diuresis periods (S_1, S_2, and S_3) in 5 experiments, of which 2 were performed on healthy animals (experiments 18 and 19), while 3 (experiments 1, 2, and 3) were carried out on nephritic animals.

Experiment	Period	Creatinine		"urine"-C.index	Creatinine clearance ml per minute = glomerular filtration	*Diuresis* ml per *minute*	Excr. %				
		serum mg%	urine mg%				Water	Sulphate	Urea	Chlorine	Glucose
Sound animal 18 . .	N	16.0	4 000	250	12.0	0.05	0.4	11	26	0.1	—
	S_1	10.8	21.6	2.00	22.5	11.3	50	107	105	35	54
	S_2	10.2	15.4	1.51	24.5	16.3	66	103	105	51	64
	S_3	10.0	19.8	1.98	15.9	8.0	50	102	101	27	35
Sound animal 19 . .	N	14.7	4 450	303	13.6	0.05	0.3	—	6	0.1	—
	S_1	12.4	35.7	2.88	21.6	7.5	35	89	88	23	—
	S_2	11.5	17.8	1.55	23.6	15.3	65	110	110	47	75
	S_3	11.7	17.8	1.52	16.7	11.0	66	106	103	47	74
Nephritic animal 1 . .	N	47.2	358	7.6	1.3	0.17	13	—	63	5.9	—
	S_1	39.1	51.3	1.31	7.2	5.5	76	96	104	64	103
	S_2	38.2	45.8	1.20	7.8	6.5	83	99	105	73	98
	S_3	37.2	43.5	1.17	7.6	6.5	86	97	108	75	110
Nephritic animal 2 . .	N	47.7	329	6.9	3.5	0.51	14.5	24	—	8.5	—
	S_1	36.0	61.0	1.61	8.0	5.0	62	102	115	49	114
	S_2	36.7	42.8	1.16	9.3	8.0	87	108	115	72	115
	S_3	35.1	36.0	1.03	6.0	5.9	97	145	129	77	129
Nephritic animal 3 . .	N	41.7	413	9.9	4.3	0.13	10.1	18.4	76	10.1	—
	S_1	33.9	37.1	1.08	10.8	10.0	93	127	112	70	127
	S_2	32.5	40.0	1.23	8.0	6.5	81	131	89	56	116
	S_3	33.3	53.5	1.60	2.5	1.6	62	129	97	32	103

For those substances, too, which are found in the tubular fluid the few figures given for the normal periods show higher values for the Excr.% in the nephritic animals than in the sound animals.

After the sulphate infusion (periods S_1 S_2 and S_3) the figures for the diuresis in the nephritic animals are found to lie between 1.6 and 10.0, somewhat lower than in the healthy animals which have 7.5 and 16.3 ml per minute.

The figures for the *glomerular filtration* are considerably lower in the nephritic animals (between 2.5 and 10.8) than in the healthy animals (between 15.9 and 24.5).

The *urine-C.index* values are quite low for the nephritic animals (right down to 1.03), and the figures for the *water-Excr.*% which in the sound animals lies between 35 and 66 during the sulphate diuresis, here in the nephritic animals shows much higher values — right up to 97.

With respect to the substances in the urine it applies to sulphate and urea that the Excr.% both in the healthy and the nephritic animals lies around 100 with a tendency to higher values in the nephritic animals than in the healthy ones.

The glucose-Excr.% only reaches 75 in the healthy animals, in the nephritic animals the figures are on a level with the figures for sulphate and urea, approaching 100.

For chlorine the Excr.% varies somewhat but the figures are considerably lower than for the other substances. In the healthy animals the values lie between 27 and 47, in the nephritic animals between 32 and 77.

Discussion.

The figures from the *normal periods* show that, corresponding to the histologically demonstrated nephritic destruction of the tubules, the ability of the kidney to concentrate the urine in any considerable degree fails. This reduction in the reabsorption applies both to the excretion of water and to the dissolved substances.

Thus the nephritis of the animals is predominantly tubular, but the relatively low figures for the filtration in the glomeruli express the fact that the glomeruli, at any rate functionally, are in some degree involved.

This appears distinctly in the *sulphate diuresis periods* in which the glomeruli have not been able to meet the demand for an

increased filtration necessitated by the sulphate infusion. That the increase in the diuresis as a consequence of the sulphate administration is less in the nephritic than in the healthy animals must be due to this fact.

But there, too, it is the conditions in the tubules which are of the greatest interest. The observed values for the urine-C.index and for the water-Excr.% have here shown that, whereas it is possible to force the kidneys of the healthy animals to send out up to 66 % of the glomerular filtrate as urine, in the nephritic animals the reabsorption of the fluid may correspondingly be reduced so much *that right up to 97 % of the glomerular filtrate, that is to say practically all the filtered amount of fluid, leaves the kidneys as urine.*

This virtually complete cessation of reabsorption in the tubules also marks the excretion of the dissolved substances sulphate, urea, and glucose.

For the no-threshold substances sulphate and urea total excretion during the sulphate diuresis is already found in the healthy animals. It is true that the figures for the excretion % of these substances are higher in the nephritic animals and — at any rate in the case of sulphate — considerably over 100.

According to the filtration-reabsorption theory it should be possible to interpret such high figures as an expression of a tubular excretion of the substances in question, but in this connection it must be kept in mind that the figures given for the excretion% factor are the result of calculations on the basis of 4 analyses and one measurement of the diuresis. Errors in analysis may here sum up, and especially with respect to sulphate the calculations must be uncertain as a result of the highly varying content of sulphate in the plasma during the experiments.

With respect to the threshold substances there still seems to be some active reabsorption of glucose in the healthy animals while it has ceased in the nephritic animals — and here too the figures for the glucose-Excr.% would seem to suggest a possible tubular excretion of this substance.

For chlorine the facts are different. Reabsorption of this substance is maintained, apparently in a not inconsiderable degree.

Even though, judging by the histological picture, an active process in the tubular cells would not seem to be probable, it turns out, nevertheless, that these tubular cells even if their reabsorption of water has virtually stopped — are still able to reabsorb

about 25 % of the filtrated chlorine. If this is really the case it will not be necessary to dismiss the possibility that the tubular cells would be able to carry out an excretion process under these circumstances.

The chief result of the experiments may be summed up in the demonstration of the fact that a profuse sulphate diuresis may be produced whether or not the tubules according to the histological picture must be regarded as capable of functioning, and that all the features characteristic of the sulphate diuresis in healthy animals are found again in the nephritic animals.

This must be regarded as a considerable support for the theory adduced, that the fundamental element in the mechanism of the sulphate diuresis is in any case a failure of the tubular reabsorption.

As stated in the conclusion in the preceding paper in this series (SCHOU 1944, 2) it has been possible, by an explanation of the sulphate diuresis consistent with the filtration-reabsorption theory, to furnish confirmation of the universal validity of this theory of kidney function.

Summary.

In rabbits with chronic tubular nephritis produced experimentally by means of potassium bichromate a. m. FRANDSEN sulphate diuresis experiments have been performed according to the principles previously laid down by the author (SCHOU, 1943).

In this way very low figures for the urine-C.index (down to 1.03) are obtained and correspondingly high figures for the Excr.% for water (up to 97 %) and for the dissolved substances here examined, namely sulphate, urea, and glucose, expressing a virtual cessation of the reabsorption in the tubules.

For chlorine, however, the ability of reabsorption is in some degree retained.

The features characteristic of the sulphate diuresis in healthy animals thus prove to apply also when the tubules are almost quite destroyed, which fact affords support for the theory that a failing tubular function is in any case the most important factor in the mechanism of the sulphate diuresis.

References.

FRANDSEN, J., Undersøgelser over nyrefunktionen ved kronisk artificie nephritis, Diss. Copenhagen 1923.
FRANDSEN, J., Skand. Arch. Physiol. 1925. *46*. 193. 203. 223.
SCHOU, P., Experimentelle undersögelser over sulfatdiuresen, Diss. Copenhagen 1942.
SCHOU, P., Acta physiol. scand. 1943. *6*. 79.
SCHOU, P., Ibidem 1944, a. *7*. 34.
SCHOU, P., Ibidem 1944, b. *7*. 183.

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Phosphate Exchange between Blood and Muscle Tissue Under the Influence of Insulin.

By

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Received 12 January 1944.

The profound researches of recent years on the subject of the intermediate metabolism of carbohydrates in the muscles have failed to clear up the mechanism of the effects of insulin. Several investigators, including LUNDSGAARD (1939), have advanced the opinion that the primary effect of insulin is associated with the passage of the glucose from the plasma and the extracellular fluid into the muscle cells. This passage is regarded as an "active" process in the sense that it is not a simple diffusion of free glucose. According to this view, the effect of insulin would be connected with living intact muscle cells and the insulin effect has not yet been demonstrable in in vitro enzyme experiments either. Nevertheless one cannot definitively preclude the possibility that insulin exerts its influence intracellularly by having a regulating effect on one of the intermediary stages in the conversion of glucose to glycogen.

It is a familiar fact that the effect of insulin interferes with the water and mineral salt balance of the muscles. Best known is the marked decrease in plasma phosphorus and potassium under the effect of insulin. According to CORI (1940) this effect is connected directly with the deposition of glycogen in the cells and it can be observed only when sufficient glucose is available to cause such a deposition. According to CORI the deposition of glycogen causes a temporary accumulation of a salt solution,

i. e. water with the ions which occur in the muscle cells, first and foremost phosphates and potassium. In our experience at this institute, however, there seems to be no parallel between the effect obtained with insulin on the phosphate concentration in the plasma and the simultaneously obtained effect on the deposition of glycogen. Even under circumstances where no effect on the glycogen deposition is observable, one sees an effect on the phosphate concentration in the plasma. Finally, LUNDSGAARD (1938) has pointed out that no effect is observable on the phosphate concentration in the plasma when the deposition of glycogen in a muscle preparation is increased by raising the glucose concentration in the blood, whereas the effect is to be seen when a correspondingly increased glycogen deposition is induced by the adding of insulin to the blood. Therefore it is possible that the effect on the phosphate concentration in the plasma is not a simple consequence of glycogen deposition, but that it is more directly connected with the primary effect of the insulin.

By means of experiments with artificially perfused hind-limb preparations in which we employed radioactive (labelled) phosphate we have made a study of the phosphate exchange between plasma and muscle tissue and the effect of insulin on this exchange.

The isolated hind-limb preparation must be regarded as a very suitable experimental object for testing the pure insulin effect. The supra-renals in this case are precluded from functioning, so that the results are not complicated by the compensatory adrenalin secretion which, according to CORI (1941), causes an accumulation of hexose-monophosphates (Embden ester) in the musculature, as a link in the processes that endeavour to counteract the insulin hypoglykaemia. The preparation consists mainly of muscle; the osseous system is scarcely very effectively irrigated by artificial perfusion. By adding radio-active phosphate to the circulating blood in such preparations and by following the changes in the content of inorganic phosphate in the plasma and in the activity, it will therefore be possible to estimate the phosphate exchange between plasma and muscle tissue. During the perfusion the radioactive phosphate will endeavour to distribute itself equally between plasma and musculature in proportion to their phosphorus content. Plasma is relatively poor in phosphates, whereas the muscles are relative rich in them. Accordingly the activity of the plasma phosphates will decrease during the

perfusion. This "dilution effect", by which we understand the decrease in activity per milligramme plasma P (plasma specific activity), is a direct measure of the exchange of phosphate ions between plasma and muscle cells. The object of our experiments was to examine whether the rate of this exchange, as expressed in the "dilution effect", is affected by insulin. At the same time we desired, by determining the absolute quantities of inorganic phosphates in plasma (and intercellular fluid), to arrive at an appraisal of the absolute quantities passing in unit time into the muscle cells, in order to compare these quantities with the simultaneously immigrated quantities of glucose. It should thereby be possible to decide whether there is any chance that the glucose migrates into the muscle cells in the form of hexose-phosphates formed by the glucose and phosphates of the plasma.

Technique.

Hind-limb preparations from normal cats are perfused artificially with about 250 ml defibrinated cat blood; glucose, 5 mg/min., is added throughout the experiment. The technique otherwise is as described by LUNDSGAARD, NIELSEN and ØRSKOV (1936). Radioactive phosphorus,[1] about 100,000—200,000 Kick in all, with considerable activity per mg P, is added in the form of neutral inorganic phosphate at the beginning of the experimental period. Plasma from the blood samples is precipitated with trichloroacetic acid; determinations of inorganic phosphates are made on the filtrates. The labelled phosphorus was determined by means of a GEIGER counting arrangement. The blood glucose concentration was followed by determinations according to HAGEDORN and NORMAN JENSEN.

Before and after the addition of insulin (or adrenalin) a sample of muscle was taken from symmetrical places on the preparation by means of ligating and cutting the anterior tibial muscle. The muscle sample was thrown into liquid air, weighed, minced, triturated and extracted with a solution of trichloroacetic acid. The filtrates were analysed for pre-formed phosphates + phosphate liberated by 7 minutes hydrolysis with ln HCl at 100° (P_7), and phosphates freed by wet ashing (P_t). The content of radio-active phosphate was also determined. Insulin ("Scott" insulin dissolved in a minimum quantity of 0.1 n HCl) was added immediately after the second blood sample was taken. Adrenalin was added continously in some experiments. Neither insulin nor adrenalin was added in the control experiments.

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[1] We wish to express our hearty thanks to Professor G. HEVESY for the radioactive phosphorus most kindly put at our disposal.

Results.

It will be seen from Table 1 that there is no specific difference between the control experiments and the insulin experiments in respect of the decrease in the specific activity of the plasma phosphate (dilution effect). This is also clear from the curves in fig. 1 showing the average values of the fall in the activity of

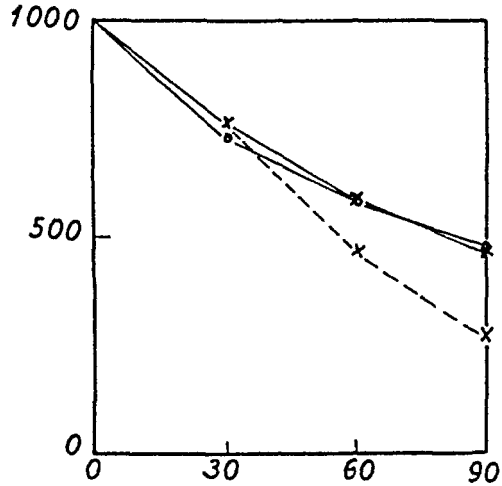

Fig. 1.

Decrease in labelled phosphorus in plasma from artificially perfused hind-limb preparations. 0 = experiments without insulin. × = experiments with insulin. The stippled curve is theoretical and indicates the calculated decrease in labelled plasma-phosphorus if the rate of exchange of phosphorus were increased two-fold.
Ordinate: Units of Activity per mg P.
Abscissa: Time, minutes.

the plasma phosphate in control and insulin experiments. The stippled length of curve represents the course which should have been described by the insulin experiments if the rate of exchange had increased twofold after the addition of insulin. Thus the exchange of phosphate ions between plasma (and intercellular fluid) and the interior of the muscle cells is not affected by insulin. This is to be seen from the insulin experiments alone. In these experiments the fall in the specific activity in the first period prior to the addition of insulin averaged 22 % and in the two periods subsequent to the insulin 22 and 20.5 % respectively.

Table 1 also shows the results of the determinations of inorganic phosphates in the plasma. The absolute quantities of phosphates migrating per period into the musculature can be calculated in

Table 1.

Variations in the activity/mg P. and inorganic phosphates in plasma of the perfusion blood in hind-limb preparations of cats — partly control preparations, partly preparations under the influence of insulin or adrenalin. The value of act./mg P for plasma are converted so that act./mg P for first plasma sample is equal to 1000.

Exper. No.	Plasma, Activity/mg P				P, mg per 100 cc plasma				Min. between blood samples
	1.	2.	3.	4.	1.	2.	3.	4.	
1. Control	1000	749	572	412	5.79	6.29	6.39	6.75	20
2. »	1000	735	640	500	6.77	7.26	7.27	7.22	—
3. »	1000	707	607	528	6.51	6.86	6.89	6.89	30
4. »	1000	697	636	576	8.28	9.14	9.20	9.20	—
5. »	1000	720	560	470	9.75	9.75	9.40	9.20	40
6. »	1000	729	571	453	7.40	7.70	7.50	7.10	—
7. Insulin 1.3 mg	1000	815	730	611	8.65	9.20	8.75	7.90	20
8. Insulin 1.7 mg	1000	780	537	526	7.58	7.60	7.93	6.48	—
9. Insulin 6.0 g	1000	818	555	490	6.68	7.15	6.89	6.38	—
10. Insulin 3.0 mg	1000	649	553	394	6.53	7.20	6.74	5.80	30
11. Insulin 1.4 mg	1000	830	640	420	5.94	5.95	4.30	2.55	40
12. Insulin 1.0 mg	1000	778	580	414	6.10	5.90	4.02	2.35	—
13. Adrenalin 1 γ/Min.	1000	750	590	532	6.91	7.28	7 28	6.91	30
14. Adrenalin 1 γ/Min.	1000	662	465	420	8.00	8.66	9.10	9.10	—
15. Adrenalin 3 γ/Min.	1000	719	588	560	8.10	8.46	8.30	7.56	—

the following manner. From the specific activity and the phosphate concentration in the plasma the total activity per 100 ml plasma or intercellular fluid can be calculated at the beginning and close of each period. By this means we obtain an expression of the absolute decrease in the activity per 100 ml plasma in each period. Assuming that the phosphate which during a period has migrated into the muscle cells had an activity corresponding to the average activity of the plasma phosphate in the period in question and the phosphate that has migrated out of the muscle cells had an activity = 0, it is possible to calculate the immigrated quantity of phosphate in each period per 100 ml plasma (and intercellular fluid). The assumption that the phosphate moving out of the muscle cells has an activity = 0 is not fully satisfied. But owing to the very great absolute quantity of phosphate in the aggregate perfused musculature (about 500 g) only very low

specific activities can be reached in the muscle phosphate within the period of the experiment. This we have made additionally certain by means of direct determinations of the specific activity of the muscle phosphate in the muscle samples. In this case, however, we have merely determined the specific activity in the fraction of the muscle phosphate that comprises inorganic phosphate, creatine phosphoric acid and adenyl pyro-phosphate. In this fraction the specific activity does not get beyond ½ % of the activity in the plasma phosphate. Even if the inorganic phosphate in the musculature may have a rather higher activity than the average activity for the fraction tested, the activity of the inorganic phosphate of the muscles must be so low that no serious error is committed by reckoning that the phosphate migrating from the muscles has an activity = 0. Previous investigations have shown that the active phosphate is very quickly built into the creatine phosphoric acid and the adenyl pyrophosphoric acid (HEVESY and HAHN 1940). The analyses of the muscle samples otherwise gave results of no particular interest; we have therefore refrained from reproducing them in detail.

The results of the calculation of the quantity of phosphate immigrated in the various experimental periods averaged as follows:

	1st period	2nd period	3rd period
Control experiments . . .	2.06	1.54	1.56
Insulin experiments	1.51	2.39	2.44

The values are mg.P per 100 ml plasma.

The increases of the phosphate immigration in the last two periods of the insulin experiments (after adding the insulin) is not accompanied by any intensified exchange of radioactive phosphorus; therefore it may solely be attributed to the observed fall in the plasma content of inorganic phosphate.

In numerous previous experiments, in which a known quantity of glucose was added to the blood in the middle of the perfusion, it was possible to calculate the total quantity of plasma and extracellular fluid at between 300 and 400 ml. in the experimental arrangement employed. Thus the absolute quantities of phosphates migrating into the muscle cells should per period (of an average duration of 30 minutes) have been about 6 mg P under conditions where no insulin was added to the blood, and a maximum of 10 mg P after the addition of insulin. The quantity of glucose assimilated per minute in the control experiments and in the

period prior to the addition of the insulin amounts to about 5 mg per minute, or 150 mg per 30 minutes. In the formation of hexose-monophosphates 150 mg glucose combines with 27 mg P. Thus it should be possible definitely to preclude that the glucose migrates into the muscle cells in the form of hexosephosphates formed from the phosphates of the plasma. After the addition of insulin about 10 mg P migrate into the muscles per period, as already stated. But after the insulin has been added the glucose assimilation amounts to about 300 mg per 30 minutes. Thus after the addition of the insulin the disproportion between the immigrated quantity of phosphates and the quantity that should immigrate if the glucose were transported in the form of hexosephosphates, is still greater than before the insulin is added.

In the four experiments in which adrenalin was continuously added to the blood during the last two periods, no definite effect was observed on the phosphate exchange between plasma and muscle any more than in the insulin experiments.

Summary.

By means of experiments on artificially perfused living hind-limb preparations of cats the authors investigated the phosphate exchange between plasma and muscular tissue. The addition of insulin produced the familiar reduction of plasma phosphate as an indication of an immigration of phosphate ions in the musculature in excess of the emigration, but otherwise no alteration in the exchange of phosphate ions between plasma and muscles.

Both before and after the addition of insulin the quantity of phosphate ion immigrating per time unit is so slight in proportion to the simultaneously assimilated quantity of glucose that a passage of the glucose into the muscle cells in the form of hexosephosphates formed from the inorganic phosphates of the plasma may be regarded as out of the question.

Literature.

CORI, C. F., Endocrinology 1941, *26*, 285.

HEVESY, G., and L. HAHN, Kgl. danske Vidensk.Selsk. Biol. Medd. 1940, *15*, 7.

LUNDSGAARD, E., Skand. Arch. Physiol. 1938, *80*, 291.

LUNDSGAARD, E., Upsala Läkarefören. Förhandl. 1939, *45*, 143.

LUNDSGAARD, E., NIELSEN, N. A., and S. L. ØRSKOV, Skand. Arch. Physiol. 1936, *73*, 296.

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The Dark-Adaptation of Mammalian Visual Receptors.

By

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The experiments on colour reception in mammals, published in preceding volumes of this Journal, consisted in following after light adaptation the recovery of sensitivity in the dark by threshold measurements in different wave-lengths. Consequently a large material of observations on dark adaptation was accumulated in this work. I have now inspected this material from the specific points of view of dark adaptation and added to it some systematic series of observations completing it in certain respects. A brief report of these results follows.

Of particular interest is that the guinea pig's eye (GRANIT, 1942) is a pure rod eye which in the experiments on the cat's retina (GRANIT, 1943) was compared with an eye having a number of cones sufficient to cause a Purkinje shift in a large percentage of the elements under the electrode. There is no Purkinje shift in the guinea pig's eye. This difference is due to the fact that the cat possesses a "carrier" of the Purkinje shift in the broad dominator band of sensitivity with maximum in 0.560μ, an element lacking in the guinea pig.

Method.

A single spike or a place giving a highly restricted discharge having been located by the microelectrode (inserted into the opened eye) the animal was light-adapted to 2,400 m. c. for 10 min. and then allowed

to dark-adapt. In the colour work the course of dark adaptation was followed by measuring the threshold for the wave-length used as standard calibration wave-length, generally 0.500 μ, which is at the top of the visual purple absorption curve. From the point of view of dark adaptation the experiments on colour were often incomplete because of their relatively short duration. Many of them were interrupted, when dark adaptation proper set in, for the simple reason that this process always involved dominance of the photosensitive properties of visual purple and thus a source of error when regarded from the point of view of the experimental problems which at that time were in the centre of interest.

In the new experiments that now were added white light instead of spectral light was used for following the decrease in the threshold during dark adaptation of long duration.

Results.

For quantitative work cats are better preparations than guinea pigs because they can be used as decerebrated animals with no more anaesthesia than is necessary for suppressing spontaneous eye movements (5—10 cc 20 % urethane). The guinea pigs received 4—6 cc urethane and, as the final threshold is very much influenced by the degree of anaesthesia, this must be the explanation of the fact that the thresholds are pushed upwards and during dark adaptation decrease far less in guinea pigs than in cats.

Some typical curves from the cat's eye are shown in fig. 1. The most striking fact is that dark adapration runs a very different course in the three lowermost curves (2—4) referring to three different experiments with white light. The uppermost curve (1), redrawn as a dashed line at the initial level of the others, was obtained by inserting an Ilford spectral red filter and making alternative threshold measurements for red light in the experiment in which curve 2 for white light in the same figure was measured. The red light (curve 1) excludes significant participation of visual purple in the process of dark adaptation. Hence, just as first shown by KOHLRAUSCH (1922) for the human eye, only the first phase of dark adaptation, the one commonly ascribed to cones, is present with this light. Curve 2 for white light, taken at the same occasion is seen not only to participate in this initial phase but also to possess a second delayed phase, dark-adaptation proper, bringing the threshold down to a level of sensitivity that only can be reached by elements activated by the highly photosensitive visual purple. The spectra of the discharge were not

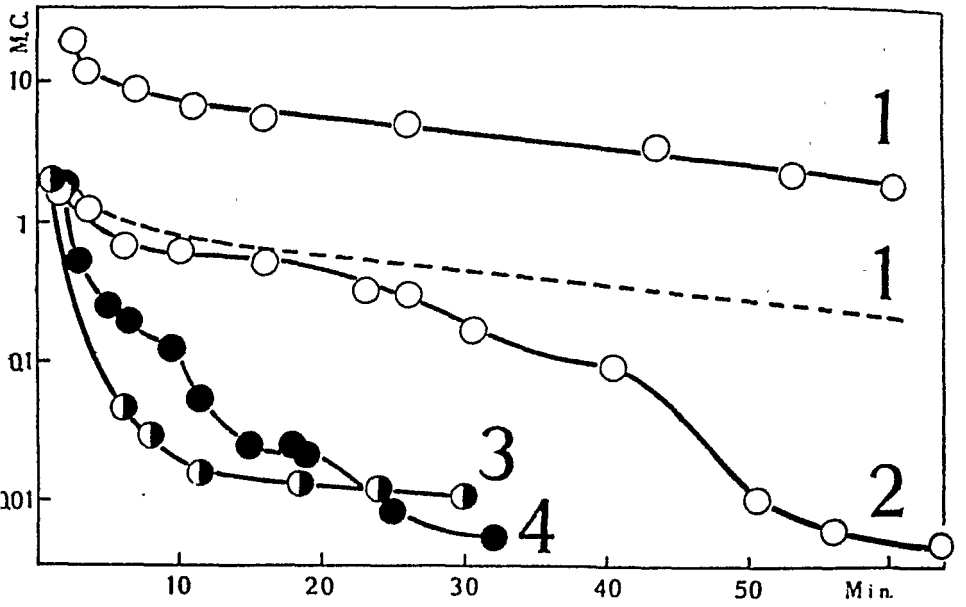

Fig. 1. Ordinates, absolute threshold in log meter candles, against time in the dark in minutes on the abscissa.

1 and 2, from the same experiment with highly restricted discharge, 1 with red filter inserted in the light beam.

3 and 4, two different experiments on dark-adaptation of single spike.

measured in this particular case, neither before nor after dark-adaptation but the colour work has shown very definitely that the delayed phase of dark adaptation always signifies that the spectral properties of visual purple become dominant.

The small initial drop of the threshold that is followed by a period of delay before dark adaptation proper sets in is not seen in all elements. Some of them, sample curve 3, show an immediate and continuous drop of threshold ending in the asymptote common for all curves. The remaining curve 4 illustrates a not uncommon step-like drop of the threshold. In this case it lasts about 10 min. before dark adaptation proper sets in.

Inspection of the material obtained in the colour work with the guinea pig shows that in this pure rod eye curves of different type also were obtained. From the point of view of a strict duplicity theory it would have seemed reasonable to expect only smoothly and rapidly dark-adapting elements such as 3 of fig. 1 in this eye and, above all, no period of delay. However, this is not the case. A period of delay before dark adaptation proper is seen every now and then in this eye too despite the absence of dominator

and Purkinje shift. Elements of this type can hardly in the cone-free guinea pig eye have been cones but must be some kind of cone-like rods. The curves from the eye of the guinea pig were just as complicated as those from the cat's eye, even though perhaps the majority could be said to be of the simple type illustrated by 3 in fig. 1.

Comment.

It is out of the question to explain all the different curves for dark adaptation of individual elements by equally many differences in the adaptive process. Pressure by the micro-electrode might be considered, but the curves 2—4 of fig. 1 all reach much the same final level of sensitivity and 2 illustrates an element still active at maximal sensitivity after more than an hour. Without excluding pressure by the micro-electrode as a source of error in some experiments it is permissible in view of such results as those presented in fig. 1 to exclude an artefact of this nature in many if not in most experiments.

All results can be very simply explained by assuming two kinds of receptors: (i) Real rods and (ii) Cones and cone-like rods. (i) The real rods dark-adapt along a smooth curve beginning without any period of delay as exemplified by 3 in fig. 1. (ii) The cones and the cone-like rods adapt quickly to a semi-stationary level representing a relatively high threshold in the dark, as shown by curve 1 for red light in fig. 1.

In fig. 2 cone-like rods are illustrated as schematic cones, real rods as rods without thereby hypothecating anything regarding differences in form between them. The difference is probably wholly on the photochemical plane. It can be seen in fig. 2 that owing to the convergence of several receptors towards the same fibre in the optic nerve (the one from which the micro-electrode records) real rods may be combined with cones and cone-like rods in different proportion. In a combination of type 3 real rods alone contribute to the element recorded from and consequently a smooth curve of type 3 in fig. 1 is obtained. In a combination of type 1 the cone-like rod (or cone) will first display its own adaptive

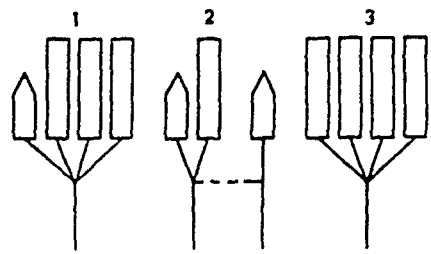

Fig. 2. See text.

properties. But after regeneration of a sufficient amount of visual purple in the real rods, *i. e.*, after a period of delay, they have recovered enough to determine the thresholds during the later phase of dark-adaptation, above called dark-adaptation proper. Again, with a great number of cones or cone-like rods and few real rods coupled to the fibre recorded from it may last very long before the latter can make their influence felt in threshold measurements.

A combination of type 2 has been drawn to illustrate that convergence also may be brought about by horizontal connexions such as horizontal cells and amacrines.

These are not the only experiments which make it necessary to assume the existence of intermediate forms between rods and cones. The work on colour reception in the same animals (GRANIT, 1942, 1943) has necessitated the same assumption.

Explanation of the different curves on the idea of convergence of a variable number of receptors of two different types presupposes that it can be shown that there is summation of individual converging receptors upon the same fibre in the optic nerve. This has been shown directly by HARTLINE (1940) with the frog's eye.

Summary.

Dark-adaptation has been followed in cats and guinea pigs by measuring the absolute threshold of single or highly restricted discharges, isolated with micro-electrodes.

The curves obtained are of different types, as illustrated in fig. 1.

The different adaptation curves can be explained by the assumption that there are two kinds of rods converging in different proportion towards the same fibre in the optic nerve, (i) real rods and (ii) cone-like rods with adaptive properties similar to those of cones.

References.

GRANIT, R., Acta physiol. Scand., 1942, *3*, 318.
—, Ibidem, 1943, *5*, 219.
HARTLINE, H. K., Amer. J. Physiol., 1940, *130*, 700.
KOHLRAUSCH, A., Pflüg. Arch. ges. Physiol., 1922, *196*, 113.

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The Exchange of Ions between Cells and Extracellular Fluid. II.

The Exchange of Potassium and Calcium between the Frog Heart Muscle and the Bathing Fluid.

By

AUGUST KROGH, ANNA-LOUISE LINDBERG and BODIL SCHMIDT-NIELSEN.

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The antagonistic effects of potassium and calcium on the frog's heart muscle have been studied repeatedly and appear well known, but opinions regarding their points of attack seem to be unsettled and vague. Until recently it was generally assumed that living animal cells were impermeable to kations. This assumption was supported by the well known fact of a very high potassuim concentration inside cells (including the heart muscle) and would involve as a necessary corollary that the effects studied must be surface effects. On the other hand several authors, who personally worked with the heart, appear to assume by implication or explicitly that an exchange takes place between the interior of the cells and the bathing fluid. Thus BOEHM (1914), who made a very elaborate and careful study of the behaviour of the frog's heart, exposed to salt solutions, emphasized the fact that a heart brought to a standstill by perfusion with sodium salt will recover when left to itself with a small volume of the solution, and showed by analysis that such a heart would give off Ca to the bathing fluid. B. KISCH (1926) states expressly, but without definite evidence, that the K effects on the muscle cells, as distinct from the effects on the stimulus formation, do not depend on a concentration gradient, but on the potassium concentration within the cells.

The recent demonstration by means of radioactive isotopes (HEVESY et al.) that monovalent kations do exchange across cell surfaces makes it worth while to utilize the frog's heart for a quantitative study of the exchange in which the results of chemical analysis can be correlated with the effects on the heart contractility.

We found it necessary to work on large specimens of frogs (R. esc. weighing at least 70 g) and as these could be obtained in very limited numbers only, we supplemented the supply with a few large specimens of toads (Bufo vulgaris).

We are greatly indebted to Mr. ARNE LARSEN, M. A. of the State School, Rønne and to Dr. H. V. BRONSTED, head of the State School, Birkerød, who supplied us with animals. In spite of the help thus freely given we have had to think twice before sacrificing each animal.

The hearts were made to work with small known volumes of suitable solutions which could be analysed for K^+, Ca^{++} and total kations, and the general arrangement was that described by MARIE KROGH (1926) or modified from it for the special purposes of the present investigation.

Details of Experimental Method.

The frog is pithed and a special silver cannula introduced into the ventricle through the bulbus cordis. The heart is completely removed from the frog, suspended below the reservoir (Fig. 1, 1) and carefully washed with Ringer. When blood-free it is enclosed in the chamber (2) connected with a Marey tambour which records the contractions on a smoked kymograph surface moving at the rate of 1 cm/min. For experiments of short duration or requiring a very small volume of fluid this simple device is utilized, but because the hearts suspended in this fashion are never tight the modification shown in fig. 2 was designed to pump the fluid leaking out back into the reservoir. In both modifications provision is also made to apply air pressure to the outside of the heart and take out practically the whole of the fluid contained at the end of an experimental period.

During each period the screw clip (3) is adjusted so as to maintain a nearly constant level of fluid in the chamber. When a period is to be closed the tap (4) is turned so as to connect with the signal instead of the reservoir. A clip is placed at (5) and pressure up to 1 m water is applied to the heart. The moment of compressing the heart is marked on the kymograph. In app. I fluid is emptied out from (1) by means of a pipette, in II the clip (7) is opened and fluid allowed to run out slowly into a sampling vessel.

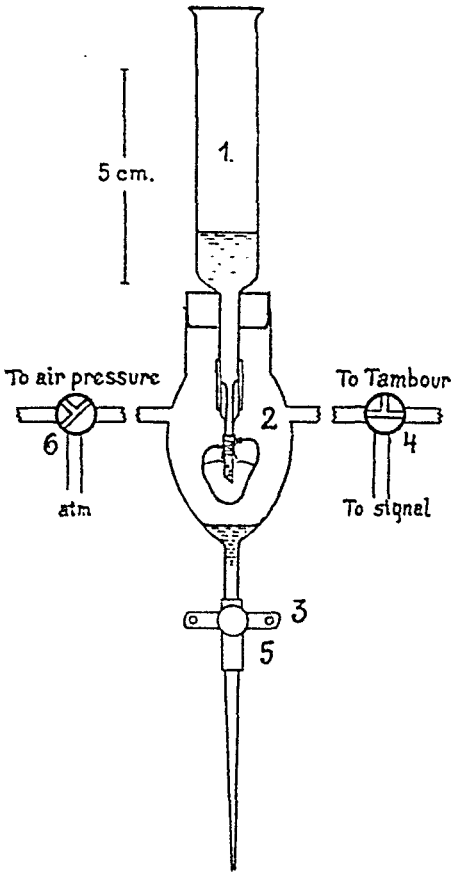

Fig. 1.

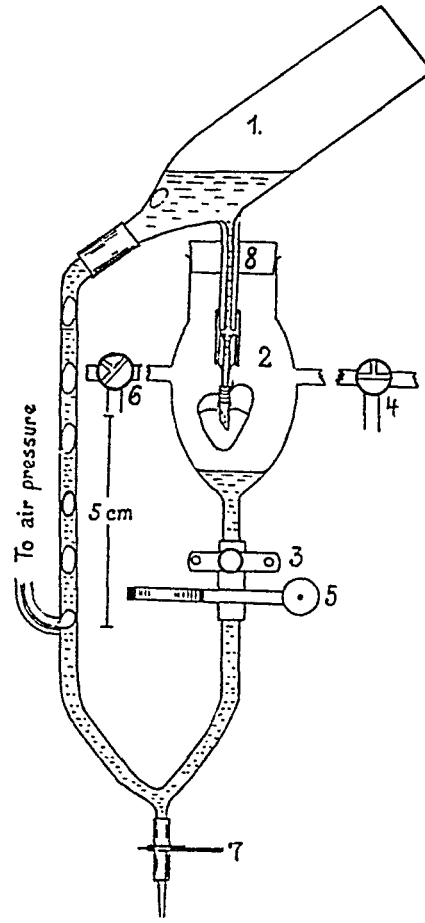

Fig. 2.

Finally the clip (5) is opened, the fluid from the chamber blown off and collected with the rest. Tap (6) is turned to connect the chamber (2) with the atmosphere.

A known volume of fresh solution is now poured in and the moment marked. The taps (4 and 6) are turned back to connect the heart chamber with the kymograph and cut it off from the atmosphere. The clip (3) is adjusted.

In cases where changes are made abruptly from one fluid to another of very different composition it is important to have a fairly accurate knowledge of the amount of fluid left over from one experimental period to the next, and this "dead space" was often determined. It is made up of 1) the fluid adhering to the glass walls of the apparatus 2) the fluid contained in the tube (8 fig. 2) and the cannula and 3) the fluid in the heart which is after compression mainly held in the extracellular spaces of the muscle proper.

It is important to remember that the heart has no blood vessels, but the meshwork of muscle fibres is sufficiently open to give easy and immediate access of the fluid contained to the surface of all the cells.

In routine determinations the quantities 1 and 2 can be kept nearly constant, while 3 is subject to variations depending upon the state of the heart. Determinations were made by adding to certain of the fluids used to feed the heart either sugar or sodium thiocyanate. In a single experiment saccharose was used, but it was found that added glucose was utilized to such slight extent by the heart that it could be safely used for these dead space determinations. Thiocyanate was generally preferred. When changes were made from a fluid containing one of these test substances to a known volume of a solution free from it analysis of both solutions gives a good determination of the degree of admixture.

After an experiment the heart is usually weighed, dried and ignited. The ash is weighed, dissolved and several determinations of ionic constituents made. In most cases these constituents are further distributed on the extracellular and the intracellular solutions, taking the first to be identical with the bathing fluid in the final experimental period.

The normal procedure is as follows. The heart is removed from the cannula, the bulbus cordis with the ligature cut off and the ventricle opened by one cut with a pair of scissors. The heart is then pressed lightly with filter paper and weighed in a small weighing bottle. 0.200 ml of the Ringer solution with which it is assumed to be in equilibrium, but containing a test substance to determine the extracellular volume, is added. As test substances we have employed sodium thiocyanate, saccharose, glucose or radio sodium. The latter substance has the decided advantage that it can be determined both before and after ignition, thereby providing a valuable control. There is reason to believe, however, that some exchange with the muscle cells will take place with all these substances except perhaps thiocyanate. After 10 minutes' gentle agitation the heart is transferred to a platinum boat and again weighed. The second weighing is in the case of the toad heart usually up to 30 mg lower than the first, and it is assumed that the difference is extracellular fluid squeezed out from the heart by the contraction induced. The differences observed on Rana hearts were quite small. The preparation is now dried to constant weight at 105°, weighed and ignited as described in the preceding paper (KROGH 1943). The ash weighed to 0.01 mg is dissolved in 1 ml normal HCl. The boat is placed in a small quartz test tube and kept with the HCl for several hours at about 65°. This treatment is necessary to dissolve the whole of the calcium present in the ash, and when it is carried out in glass significant amounts of alkali may go into solution. The solution is made up to 4 ml with water and usually determinations of K, Ca and total kations carried out on suitable aliquot parts. When radio sodium was used for determination of extracellular fluid this is again determined on the ash solution. The agreement has been very satisfactory showing the same change in extracellular fluid as had been found by weighing.

In a few cases phosphoric acid was determined and in a few others

the hearts were used whole for chlorine determinations according to REHBERG. A few determinations of Ca, K and total kations were made on blood of frogs and toads.

Analytical Procedures.

Potassium is determined mainly as described in the preceding paper by A. KROGH (1943), but with the following alterations. It was found that in solutions containing only K, Na, Ca, Cl and HCO_3 the ignition could be omitted when they had been in contact with the heart for a couple of hours or less, while solutions containing sugar or thiocyanate must always be ignited. Colorimetric tests showed that the colour of iodoplatinate develops much more slowly than formerly supposed and is in our solutions safely complete only after 5 hours at 38—40° or 10 hours at room temperature. At the high tp. platinum may begin to appear a few hours later, so that on the whole it is safer to leave the samples at room tp. and titrate the next morning. With this precaution results have been definitely improved and are usually reliable within 2 %. Total kations, glucose, saccharose and thiocyanate are determined as before.

Calcium is determined according to SOBEL and SOBEL (1939) with slight modifications. The calcium is precipitated as oxalate, washed in a centrifuge tube with ammonium oxalate, converted in an electric oven at 430° C into carbonate, dissolved in hot boric acid, diluted and titrated with HCl to the pH of boric acid. The accuracy is about $^1/_2$ % when 1 ml 1 mM solutions are determined. Phosphate is determined according to the method of LOHMANN and JENDRASSIK as described by KJERULF-JENSEN (1942).

In the following all concentrations are given in millimolarity (mM) and quantities in microequivalents (μE). 1 μE of Na and K is 0.001 millimole, but of Ca only 0.0005 millimole.

The Inorganic Constituents of Blood and Hearts.

About the middle of June we made analyses of the serum from two frogs and two toads, all recently caught, with the following results in millimolarity.

	Rana		Bufo	
Weight g	70	72	76	60
K	3.9	3.2	5.1	5.75
Ca	2.9	2.3	2.4	2.5
Kations	138	134	115	124

Analyses were again made in December on the mixed serum from two frogs caught in September. They showed a lower K concentration 2.0, Ca 2.5 and kations 126.

The concentrations of K and Ca are definitely higher than those generally used in experimental solutions, but the experiments have shown that the differences are of no consequence for our purposes, and it was decided to stick to the Ringer solution employed from the start.

The general results of about 20 analyses of hearts from frogs and toads are as follows. The hearts of animals weighing from 60 to 110 g show weights ranging from 150 to 380 mg, and the correlation between body size and heart weight is not very pronounced. The frogs with an average weight of 72 g showed an average heart weight of 180 mg or 0.25 % and 6 toads weighing on an average 85 g had a heart weight of 270 mg or 0.32 %. The dry substance of the hearts varied from 12 to 17 % with an average of 14.7 % and the ash from 0.9 to 1.1 % with an average of 1 %. In the routine analyses K^+, Ca^+ and total kations are determined. On 4 hearts special determinations of Cl^- were made as follows.

The heart from one frog and one toad were treated with the ordinary Ringer, the other two with a corresponding nitrate solution renewed twice; all were directly dissolved in nitric acid with silver nitrate and the chlorine determined according to REHBERG. The results were:

	Heart mg	Water 84.2 % mg	Cl^- µE	Extracellular			Intracellular		
				Water 25 % mg	Cl^-		Water 75 % mg	Cl^-	
					mM	µE		µE	mM
R 70 g . . .	209	176	1.61	44	0.8	0.035	132	1.57	12
R 72 g . .	224	188.5	12.80	47	127	5.96	141.5	6.84	48
B 76 g . . .	233	196	2.73	49	0.8	0.04	147	2.69	18
B 60 g . . .	151	127	7.55	32	127	4.06	95	3.49	37

The values for the weight of the heart, the total Cl and the Cl concentrations in the extracellular fluid are directly determined, the others are calculated, assuming average values for the amount of dry substance and the extracellular volume of such hearts. The final results are uncertain therefore, but the existence of chlorine inside the cells cannot be doubted, and it is at least very probable that some Cl was removed from the cells by the nitrate treatment.

On some other hearts phosphate was determined along with the kations and the concentration found to be some 10 % lower

than that of potassium, and from all the determinations combined the approximate weight of the ash can be figured out as a check on the determinations and is found to agree within about 10 % of the actual. This check is of some importance, because the results of the analyses are rather startling and require all the support they can obtain.

It is a necessary consequence of the free permeability of the cells for water and the absence of any mechanically resistant cell wall that the osmotic concentrations inside and outside the cells must always be the same. In the extracellular space the osmotic concentration is made up practically exclusively of the ions of the Ringer solution and amounts to about 130 mM kations and an equal concentration of anions. Inside the cells part of the pressure may be made up of organic substances and the concentrations of ions may be lower than outside, but not higher.

When however the total kations found in a heart by analysis are calculated as dissolved in the water content determined the concentration invariable becomes higher than 130 mM and lies generally between 150 and 170 mM, rising occasionally even to 200 mM, while the actual amounts vary according to the size and water content of the hearts between 18.4 and 49.6 μE.

This must mean that only a certain proportion of the ions are free to exert an osmotic pressure, while the rest are firmly bound in some way.

The water of the heart is partly extracellular, partly intracellular. In the extracellular fluid, which in these experiments is a Ringer solution, all the ions are known to be free and the apparent kation concentration must be even higher for the intracellular solution. In a number of our determinations we have endeavoured to distinguish between the two solutions by adding, as described above, a known amount of a test substance which should become distributed in the extracellular solution only. We have no proof that the test substances employed viz. thiocyanate, glucose and ^{24}Na cannot penetrate into the cells; in fact we know that the two latter will do so, even if slowly, and we have no proof further that they become evenly distributed in the whole of the extracellular fluid in the course of the 10 to 15 minutes allowed; but when using two test substances together we have obtained substantially the same result for both and when taking consecutive samples of the fluid bathing the heart muscle we have found it to become practically constant with regard to the test substance

after about 5 minutes, but on the other hand we have found the extracellular fluid to vary considerably in amount from one heart to another viz. between 12 and 40 % of the total with an average of 28 % (10 hearts).

When the extracellular kations, calculated from the volume of extracellular solution and its composition, are subtracted from the total and the rest taken to be dissolved in the intracellular water the apparent concentration is further raised to values between 155 and 240 mM.

The potassium quantities found in the hearts show variations in relation to the size between 8 and 20 μE, and the concentration calculated for the heart as a whole varies only between 50 and 80 mM with an average of 62 $\pm$ 3.4 (8 determinations). Almost the whole of the potassium is intracellular and the calculated intracellular concentration is on an average 82 mM $\pm$ 5.2. The fact that the error on the average is increased does not inspire confidence in the determinations of the extracellular fluid volume.

The calcium content of the hearts is much smaller, but extremely variable, namely from 0.7 μE in a heart of 145 mg to 2.36 μE in another of 261 mg. The calcium in the extracellular fluid is always a very small quantity, and the intracellular calcium is possibly or perhaps probably chemically bound to tissue elements. Calculated as free calcium ions the concentrations would vary from 3—7 mM.

In the kation determinations the possible small quantity of magnesium present does not enter, and the differences between total kations as determined and (K + 2 Ca) is therefore sodium which makes up from about 1/2 to 2/3 of the total within the cells.

It follows from the above that either potassium or sodium or both must be *partly* combined in such a way as not to exert any osmotic pressure. HEVESY and HAHN (1941, a, b) have shown that when tissue cells (muscle, heart, liver, brain, red corpuscles) are exposed to an extracellular fluid containing ^{42}K, but otherwise normal, a fairly rapid exchange takes place, but remains incomplete, involving less than $^1/_2$ of the total potassium. Experiments cannot be prolonged indefinitely because of the rapid deterioration of ^{42}K (half life 13.5 h) but there is no doubt that more than half the potassium exchanges much more slowly than the rest, if it exchanges at all. In the working muscles of swimming rats the exchange is speeded up, but the equilibrium reached remains the same.

We have made one corresponding experiment on a frog's heart and were prevented from doing more, when the Institute for Theoretical Physics was suddenly seized by the Germans. The experiment was carried out as follows. At 12^{55} the heart was supplied with 6.2 ml Ringer with 3.0 mM K of which 11.4 μl registered 256 kicks/m. 10 minutes later the activity had dropped to 224, at 13^{35} it was 176 and from 14^{43} onwards to 18^{30} it remained constant at 164. The heart was dried and ashed, but the ash unfortunately lost. On the basis of the many determinations made the total ash was estimate l at 2.3 mg with 12 μE potassium.

The initial activity determined corresponded for 6.2 ml with 3.17 mM K to 19.65 μE with 139 500 kicks/m or 7 100 kicks/μE/m. During the period 0.1 ml corresponding to 0.3 μE and about 2 000 kicks were removed in samples.

At the end the concentration had dropped to 2.85 μE or a total of 17.4 μE with 88 000 kicks or 5 050/μE/m.

The difference between 137 500 and 88 000 = 49 500 must have entered the heart and corresponds to 9.8 μE.

The bathing fluid has lost 19.65 — 0.3 — 17.4 = 1.65 μE to the heart and the rest about 8.1 must have been exchanged. This is about 2/3 of the total quantity estimated. So far as it goes this estimate points to a fraction of the potassium as being combined in such a way as not to exchange readily and not exerting any osmotic pressure.

When a frog's heart is exposed for a few hours to a potassium free salt solution potassium is eliminated from the muscle cells and is replaced by sodium, but the concentration of K in the bathing fluid can be increased considerably (up to 6 mM) without any, but the slightest effect on the muscle cells in the heart. This is well illustrated by the following analyses of 4 hearts:

Bathing fluid	Weight mg	Ash %	Water content mg	Kations		K		Ca		Extracellular fluid		
				μE	mM	μE	mM	μE	mM	Kation mM	K mM	Ca mM
NaCl	162.7	0.93	142.6	27.1	190	3.2	*22.5*	1.14	4	132	0.06	0.02
R	189.2	0.97	163.6	24.0	147	9.9	*60.5*	1.36	3.5	135	1.5	1.0
R	212.8	0.95	185.0	29.6	160	11.6	*63*	0.92	2.5	136	1.5	1.0
Conc. R	145.0	0.95	125.5	18.4	147	8.1	*64.5*	1.30	5.2	132	5.85	3.91

The composition of the solution with which the hearts were, at least approximately, in equilibrium, is given in the last 3 columns. It is seen that the effect of a low potassium concentration is very pronounced while that of a very high is doubtful. No effect is noticeable on the calcium content of the hearts. It is evident from the kation figures that the potassium lost by the heart in NaCl was fully replaced by sodium.

The Reversible Exchange of Potassium and Calcium between the Heart and the Bathing Fluid.

It is possible not only to remove potassium (and calcium) from a heart to a bathing fluid with a low concentration of these constituents, but the process is reversible, and the ions are absorbed again from normal or more concentrated Ringer solution. The first heart in the table above was treated during 4.5 hours in all with an isotonic solution of NaCl + $NaHCO_3$, renewed 5 times. It lost per minute at first 0.074 μE K^+ and finally 0.004 μE, or 7.4 μE in all. The calcium loss recorded was 1.0 μE, but in spite of this the analysis of the heart at the end showed a normal Ca content.

In another experiment of this type a heart (R. esc. 70 g) was treated from 12^{30} to 18^{30} with 4 changes of 10 ml sodium solution. The concentrations found were:

	K mM	Ca mM
13^{30}	0.48	0.067
14^{38}	0.46	0.024
16^{47}	0.26	0.046
18^{30}	0.18	0.013

This corresponds to a total loss of about 11 μE K and 3.0 μE Ca. During the period the activity of the heart diminished very gradually and finally stopped altogether. This heart was then washed twice with normal Ringer and treated with the same for 6 hours. The washing caused an initial strong contracture, and later a very slight recovery of the activity was noted. Only a small reabsorption of K and Ca (less than 2 μE K and less than 1 Ca) could be found by the analyses. The heart evidently was too far gone.

In a third experiment a heart beating vigourously under normal Ringer was given NaCl + $NaHCO_3$ + 0.1 Ca for 47 minutes and lost 4 μE K^+ or 0.085/min. During the next period with normal

Ringer + 100 mg % glucose it absorbed 2.8 μE or 0.043/min. Under NaCl + $NaHCO_2$ alone without Ca and glucose it lost 4.3 μE or 0.083/min. Again under Ringer with glucose it absorbed 3.2 μE, 0.053/min. Washed out for the third time with NaCl + + $NaHCO_3$ it lost 2.7 μE, 0.044/min. Exposed next to a concentrated Ringer with 4.5 mM K instead of 1.5, but without glucose it absorbed 2.0 μE, 0.042/min. and finally under normal Ringer with glucose 2.3 μE, 0.038/min. The net loss calculated was 0.7 μE and it is seen that the rate of loss or absorption is not visibly affected by the presence or absence of glucose or calcium nor by an increase of K^+ in the bathing fluid to thrice the normal value. During the whole experiment the activity of the heart was scarcely affected.

When potassium is given off to a solution an equivalent amount of sodium (so far as can be judged) enters the cells, and both these processes take place in accordance with the concentration gradients, but when potassium is absorbed back into the cells the ion movements are directed against the concentration gradients. To avoid misunderstanding it is necessary to emphasize that the movements of ions both passive and active are, of course, not confined to the conditions in which their resultant is of a measurable size, but take place all the time at a rate which can only be measured by means of radioactive isotopes.

Eliminations and reabsorptions of calcium take place also when calcium concentrations in the bathing fluid are lowered and raised, but to a much smaller extent, corresponding to the small quantity of calcium present in the heart. The results with calcium are much less regular than with potassium and there is often a net gain or loss which we cannot explain. We give as an example the experiment of June 16 made on a toad of 98 g. The dead space in this experiment was unusually large and variable, because the atria retained a variable amount of fluid. It was however determined at each change from one solution to another.

After being bathed with Ringer for about an hour the heart was given Ca free Ringer and lost in 18 minutes 0.32 μE. In three consecutive periods with normal Ringer lasting 7, 12 and 60 minutes it took up in the first two respectively 0.16 and 0.76 μE, but lost in the last 0.28. Again in calcium free Ringer it lost 0.42 μE during 18 minutes which it failed to regain during 1 hour in Ringer — while it lost finally 0.90 μE during 17 minutes in the calcium free solution. The net loss amounted to 1.0 μE.

Nothing can be said about the gradient, because the calcium in the heart may not be in solution at all, but may be chemically bound to organic cell constituents.

When a heart is treated repeatedly and for several hours with Ca free solutions the loss gradually diminishes and some Ca is evidently very firmly held in the heart muscle.

The Activity of the Heart in Relation to the Composition of the Bathing Fluid and the Rate of Exchange with the Muscle Cells.

The problem to be discussed here is limited to the dependence of the ventricle contractions and tonus on the calcium and potassium ions as directly observed and shown in the curves obtained.

When the absolute and relative concentrations of these two ions in the bathing fluid are varied within certain limits the behaviour of the heart remains normal, and the exchanges taking place between the heart muscle and the fluid are generally small, but when concentrations are varied beyond these limits the contractility and tonus become altered and definite ion exchanges take place between the fluid and the cells. We have mainly studied the changes due to reduced concentrations.

When the bathing fluid is changed from complete Ringer to 3—10 ml of a solution of sodium alone the ventricle beat is either reduced to a minimum of less than 1/10 its former volume or stops altogether within a fraction of a minute. The rhythm is usually reduced, but may be maintained. The process of recovery begins almost at once, and with a fluid volume of 6 ml may reach a constant level in less than 10 minutes, but may also take longer, and during this period both K and Ca are given off from the heart to the bathing fluid. When the sodium solution is renewed the process is repeated and the recovery usually takes longer, but at the next repetition the contractions may become vigourous almost at once, but with a slow and usually irregular rhythm. At this point the concentrations of K and Ca are well below 0.05 and 0.005 mM respectively but rise again, in one case to 0.26 and 0.046. When the process of washing out is continued in this way, say every hour, both the amplitude and the rhythm are gradually reduced to 0 and in the single case in which we brought a heart to this point of exhaustion we found that it would not recover when supplied with Ringer.

When at any stage during the sodium treatment, except perhaps the very first, the solution is replaced by Ringer or even Ringer with a double K content the first effect is a calcium contracture which only gradually relaxes. This fact was strongly emphasized by BOEHM.

A heart exposed to a potassium free solution containing the normal concentration of calcium may beat normally for one or two minutes and then become slow and somewhat irregular. When the solution is renewed the irregularity increases, and we have observed a tendency for large beats at a slow rate appearing in groups, separated by pauses of increasing length up to many minutes before the heart finally stopped beating altogether. When the solution was replaced by complete Ringer single large beats appeared after a minute and the heart required several minutes to become approximately normal.

The reactions to changes in calcium concentrations are much more abrupt. When the heart is supplied with calcium free Ringer there is usually an immediate drop in amplitude which in some cases recovers somewhat during 1—2 minutes and then dwindles again almost to zero in a few minutes more. In other cases the contractions become minimal at once and remain so. When the heart is brought back into normal Ringer the beats are normal from the start, which is almost immediate.

From the observations here briefly recorded it is concluded that 1) the action of K and Ca on the contractility is mainly located in cell surfaces, 2) that both ions can be to a certain extent replaced from the interior of the muscle cells as well as from the bathing fluid, 3) that replacement from the cells is a fairly rapid process in the case of potassium, but much slower in the case of calcium, while the replacement from outside is practically instantaneous in the case of calcium and is retarded in the case of potassium, because the ions pass on into the cells.

The conception was formed that the action depends on a certain fraction of the ions held adsorbed or otherwise combined in the cell surface, and it was decided to attempt a determination of the quantities so combined in the case of calcium where the relatively slow exchange with the interior should make it possible. The possibility was further considered that calcium and potassium might be combined to the same active surface points, in which case it might be possible to drive out calcium by a surplus of potassium.

In these experiments the simple arrangement I was employed with just so much fluid as would be required for the analyses. From this a sample could be taken with a pipette and just afterwards the concentration of Ca or K altered by adding 0.1 ml of a fairly concentrated solution with a syringe. The contractions could be observed and recorded all the time and a final sample taken after 1—2 minutes. The action on the contractions was always immediate, but the analyses failed to show that any measurable quantity of Ca would enter into combination or be driven out into the solution during the experimental period.

We reproduce a few periods from a typical experiment. 1. The heart of a R.esc. was beating weakly (0.02/50) in a fluid containing 0.3 mM K with no calcium added. A sample taken showed a content of 0.03 mM Ca. The 3.5 ml fluid left in the apparatus contained therefore 0.21 μE. To this was added 0.1 ml isotonic fluid containing 1.35 μE Ca. The contractions were immediately increased and became constant at about (0.1/40) after 0.2 minute. The calcium found after 1.5 min. showed a concentration of 0.211 mM or 1.52 μE in all — a deficit of 0.04 μE. The next period, which was exactly similar, showed a surplus of 0.02 μE.

Later in the same experiment the heart was working regularly on a Ringer solution containing 0.3 mM K and 0.2 mM Ca (0.22/30).

The analysis showed a Ca content of 0.141 mM or 0.988 μE for the 3.5 ml solution. To this was added 0.1 ml of a solution containing 49.5 mM K and 0.2 mM Ca raising the K concentration in the apparatus to 1.67 mM and leaving the Ca practically unaltered. The contractions were immediately reduced to the vanishing point, and the analyses after 1.3 min. showed a calcium deficit of 0.08 μE. The potassium added failed to liberate any calcium.

These results do *not* show that the active ions cannot be in combination at certain points in the cell surfaces, but they do show that the quantities which may be so combined are too small to be estimated or even detected by our analyses.

In a very important paper, published in 1941, but only recently come to our notice, BOYLE and CONWAY studied the equilibrium between frog's sartorii and immersing solutions varied in different ways. They are able to describe quantitatively by theoretical equations the changes in composition and quantity of the intracellular fluid phase, brought about by varying the potassium content and total concentration of the immersing fluid which is pre-

sent in such large excess that its composition could be considered constant throughout. The necessary assumptions, verified by the experiments when the extracellular potassium concentration is above a certain limit, are that the fibre surface is impermeable to the sodium of the immersing solution and to a high concentration of complex anions, mainly phosphoric esters, found inside the fibres, while it is permeable to the smaller ions of K^+ and Cl^-.

It is shown both theoretically and experimentally that the volume of immersed sartorii remains practically constant with additions of KCl up to 300 mM, when the sodium concentration is kept constant, while the intracellular K concentration is increased and always remained higher than the extracellular. When on the other hand the total external concentration is kept constant and K substituted progressively for Na up to 100 mM the muscle swells, K is accumulated, but the concentration remains constant and is always above the external.

In ordinary Ringer and at all K concentrations below 10 mM at 2° C and below 29 mM at room temperature the muscle loses potassium and sodium enters. The authors give good reasons for the assumption that this behaviour is due to unphysiological conditions, and that the limit for the impermeability to sodium lies much lower in the living organism. On the other hand it seems doubtful that the muscles could retain their contractility in the more concentrated solutions employed.[1] In a paper to be published shortly by HOLM-JENSEN, KROGH and WARTIOVAARA it will be shown that the protoplasmic membrane inclosing the sap in the cells of the alga Tolypellopsis remains almost impermeable to ^{24}Na after treatment with formaldehyde or heat (50° C). It is a fact further that the normal muscle cells may contain Na in *variable* amounts (MOND a. NETTER 1932), that some Na penetrates into muscle cells during work and is again *eliminated* during recovery (FENN et al. 1938) and that sodium is reversibly absorbed in a K free solution (STEINBACH 1940). We think it possible to conclude that in truly physiological conditions the accumulation of potassium in muscle cells can be explained as a physico-chemical process, while sodium is kept out of the cells by an active process involving expenditure of energy.

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[1] As a matter of fact experiments which Dr. F. BUCHTHAL kindly carried out for me showed that at all the higher K concentrations at which BOYLE and CONWAY found the remarkable impermeability to sodium the muscles had lost their contractility completely and irreversibly.

In the case of the frog's heart muscle it is definitely known from the experiments described and will be abundantly confirmed in the adjoined paper that the fibres are not impermeable to sodium, but it is tempting to assume that sodium is actively thrown out from the cells, while the potassium concentration is regulated by simple chemical forces. It appears to us, however, that such an assumption is incompatible with our observation that from concentrations of about 1 mM in the bathing fluid up to about 6 mM the inside concentration remains practically unaltered.

We seem to be forced to the conclusion that in the heart there is an active transport both of K^+ and Na^+ regulating the cellular concentrations, but unable to overcome concentration differences above a certain value, because the rate of diffusion exchange is rather high.

We are indebted to Dr. Ib HOLM-JENSEN who did some of the numerous analyses, especially the CNS determinations.

Summary.

Methods are described for studying the exchange of ions between the heart muscle of the frog and the bathing fluid along with the muscle contractions and for estimating the ion concentrations within the muscle cells.

Analyses of the serum of a few frogs and toads have given values for potassium and calcium which are definitely higher than those used in Ringer solutions, but the differences appear to be of no consequence.

Although the kation concentration of the bathing fluid is about 130 mM the total kations of the heart muscle dissolved in the quantity of water present will produce a higher concentration (about 170 mM), and evidence is presented to show that a fraction of the potassium which averages 82 mM in all, is chemically combined, so as to exert no osmotic pressure, and not readily exchangeable with ^{42}K.

The calcium concentration is small (3—7 mM) and it seems probable that the calcium is mainly present in chemical combination.

A large fraction of the potassium and calcium present in a heart can be removed by repeated treatment with small volumes of sodium solutions, and the quantities thus lost are reabsorbed from

normal Ringer, if the washing out has not been carried too far. The reabsorption of K^+ is not accelerated by higher concentrations. When potassium is lost sodium enters the cells and is again driven off during the reabsorption process.

The response of a heart to a potassium free solution is gradual and takes a few minutes to develop, during which the heart becomes slow and somewhat irregular. When the solution is replaced by complete Ringer it again takes some minutes for the heartbeat to become normal.

The response to changes in calcium concentration is much more abrupt. It is concluded that the action of K^+ and Ca^{++} are mainly located in cell surfaces, that both ions can be to a certain extent replaced from the interior of the cells and that the replacement of Ca is much slower than that of potassium, while calcium from the bathing fluid acts almost at once.

On the assumption that the effects of potassium and calcium are due to ions adsorbed or otherwise combined at the cell surfaces an attempt was made to determine the quantities adsorbed in the case of calcium, but these attempts failed.

It is concluded that the heart muscle cells have the power of actively transporting potassium ions from the outside into the cells and sodium ions in the opposite direction.

References.

Boehm, Arch. exp. Path. Pharmak. 1914, *75*, 230.

Boyle, P. J. and E. J. Conway, J. Physiol. 1941, *100*, 1.

Fenn, W. O., D. M. Cobb, J. F. Manery and W. R. Bloor, Amer. J. Physiol. 1938, *121*, 595.

Hahn, L. and G. Hevesy, Acta Physiol. Scand. 1941, *2*, 51, 1942, *3*, 193.

Hevesy, G. and L. Hahn, K. D. Vid. Selsk. Biol. Medd. 1941, *16*, 1.

Holm-Jensen, Ib, Krogh and Wartiovaara, Acta Bot. Fennica 1944.

Kisch, B., Handb. norm. path. Physiol. 1926, *VII 1*, 828.

Kjerulf-Jensen, K., Fosforyleringsprocessens betydning for resorption og assimilation. Diss. Copenhagen 1942.

Krogh, A., Acta Physiol. Scand. 1943, *6*, 203.

—, and A.-L. Lindberg, Ibidem. 1944, *7*, 238.

Krogh, Marie, Physiol. Papers dedicated to prof. A. Krogh; Copenhagen. 1926, p. 133.

Mond, R. and H. Netter, Pflüg. Arch. ges. Physiol. 1932, *230*, 42.

Sobel, A. E., and B. A. Sobel, J. biol. Chem. 1939, *129*, 721.

Steinbach, H. B., Ibidem 1940, *133*, 695.

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The Exchange of Ions between Cells and Extracellular Fluid. III.

The Exchange of Sodium with Glucose in the Frog's Heart.

By

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Received 20 January 1944.

In 1913 it was shown by SAKAI that the frog's heart can work on a solution in which a large part of the sodium chloride is substituted by glucose in aequiosmotic concentration. He found that the heart could work for a long time on a fluid in which instead of 6 ‰ NaCl + 1 ‰ $NaHCO_3$ the heart was supplied with 1 ‰ NaCl + 1 ‰ $NaHCO_3$ and 31.5 ‰ glucose or expressed in molarities 87.5 mM Na and Cl out of 114 were replaced with 175 mM glucose. Then and later it has been tacitly assumed that this fluid was simply isotonic with the heart muscle cells and that no exchange would take place, but in view of the fact that these cells are undoubtedly permeable both to sodium, as shown in the preceding paper, and to glucose, which must penetrate to serve as a source of energy, the conception of simple isotonicity without any exchange could not appear selfevident to us.

A small number of experiments carried out with the experimental and analytic technique described in the preceding paper, but unavoidably limited by lack of animal material, showed conclusively that a very considerable exchange will take place.

Our glucose Ringer (GR) is made up of 3.15 g glucose, ½ ml 20 % NaCl, 2 ml 5 % $NaHCO_3$, 1 ml 150 mM KCl and 1 ml 100 mM $CaCl_2$ with redistilled water to 100 ml and comes very close to

that employed by SAKAI. The total osmotic pressure is that of a 124 mM NaCl solution of which 88 mM is replaced by the glucose.

In the first experiments we used the apparatus arranged for fluid circulation (App. II of the preceding paper) charged with 6.2 ml solution, and the heart worked for almost 5 hours on 4 changes of GR. When this was first supplied the amplitude of contractions were reduced to $^1/_3$, but recovered in 15 minutes. After 40 minutes the frequency was suddenly reduced and the amplitude correspondingly increased, and during the remaining periods in which the solution was exchanged with fresh GR both amplitude and frequency were somewhat irregular, but not more than is sometimes seen in normal Ringer. The analyses of the bathing fluid showed slight and irregular variations in the K^+ content, and a definite increase in the kation content from the 33.3 mM of the GR to a maximum after 2 hours of 34.7 mM with a final value of 33.6. The calculations, which owing to the large volume of fluid and the 1 % mean error on the kation determination cannot be very accurate, showed 6 μE of kations to be given off from the heart, while 0.7 μE K were absorbed.

The heart weighing 190 mg with 164 mg water of which 46 were determined as extracellular gave only 1.15 mg ash (0.6 % against the normal 1.0) and the determinations showed a content of 6.4 μE K and 7.6 μE kations. Distributed on the extracellular and intracellular fluid this gives for the latter 6.3 μE K and 6.0 μE kations corresponding to a concentration of 51 mM. In other words all the intracellular sodium was eliminated. Even when the possible errors on this determination are estimated at their maximum the intracellular sodium concentration at the end of the experiment must have been lower than the extracellular.

An attempt to repeat this experiment on the heart of a R.temp. was unsuccessful. The sodium concentration in the heart was reduced in 3.7 hours only to 80 mM.

In a second experiment we endeavoured to measure the kation changes more accurately by reducing the fluid volume to 2 ml, to show that the elimination of sodium is reversible and finally to demonstrate the uptake of glucose into the muscle cells by estimating the quantity eliminated in a subsequent period with normal Ringer. In order to reduce the amount of solution required for analysis the K concentration of the solutions was increased to 3 mM.

After having worked for 10 minutes on Ringer the heart was washed out during 10 minutes with 3 × 2 ml GR and then left to work with this solution for 83 minutes. During this period the kation concentration in the fluid (2.08 ml) rose from 40.7 mM to 44.1 corresponding to an elimination of 7 μE of which 0.15 μE was K^+.

The effect of the GR on the contractions was very similar to that observed in the first experiment, but it took over an hour before the heart became definitely irregular and slow.

The heart was now washed 10 minutes with 3 × 2 ml R and left with Ringer for two periods of 45 min. and 55 minutes. During the first of these 2.3 mg glucose (corresponding to 6.4 μE NaCl) was eliminated raising the concentration to 107 mg %, while the kation concentration of the fluid was reduced from 130.5 to 128.3 corresponding to an uptake of 4.6 μE. In the second period the changes both in glucose and kations were so small as to be within the limits of error (+ 0.1 mg glucose and + 2 μE kations of which 0.8 μE was K^+).

In a final period on GR of 80 minutes duration there was again an elimination of 4.3 μE kations of which 1.6 were found as K^+.

During this period the amplitude of the ventricle was at first very much reduced and took over 20 min. to recover. It was then somewhat slower than with Ringer, but perfectly regular for about 10 min. before it became increasingly irregular both as to rhythm and depth.

The heart of this frog weighed 150 mg and gave 1.19 mg ash (0.8 %). As was to be expected a rather larger quantity of sodium was left in the heart, the final intracellular concentration being calculated as 34 mM which is not significantly lower than the extracellular.

In a third very similar experiment which gave substantially the same main result it was attempted to determine the dead space in the heart simultaneously with thiocyanate and with ^{24}Na, because the results so far obtained made us suspect that even in very brief periods sufficient ^{24}Na might exchange with the muscle cells to vitiate the results.

After emptying the system as completely as possible 1 ml Ringer containing both thiocyanate and ^{24}Na was added and samples taken at short intervals. The initial concentrations of both substances being taken as 100 the following results were obtained:

Time	1	2.5	3.5	6	minutes
^{24}Na	96.8	92.5	86.5	86.8	
CNS		91	92	92	

If the dead space is calculated from the results after 3.5 and 6 minutes it works out as 87 μl for CNS and 154 for ^{24}Na. A similar experiment made while the heart was working on GR and with a solution corresponding as closely as possible to the normal GR gave:

Time	9	2.5	4.25	6	minutes
^{24}Na	94.3	84.5	83.1		
CNS	94	93	89	89	

The dead spaces deduced from these figures are 124 μl for CNS and 190 for ^{24}Na. The exchange of ^{24}Na with the cells cannot be doubted. This heart was beating at the very slow rate of 10/min., because the venous sinus had accidentally been cut off. In a heart beating normally a complete mixing of the dead space fluid with the solution poured in is attained in a definitely shorter time.

Finally an experiment was made in which saccharose was substituted for glucose, the idea being that saccharose should be unable to penetrate into the cells.

In this experiment it was attempted to obtain some additional information by weighing the heart at short intervals. If sodium salt could leave the cells without being replaced by saccharose the weight ought to fall, because water should be sucked out osmotically.

Before each weighing the heart was compressed by air at 1 m water pressure, gradually applied. All fluid above the cannula was sucked out, the pressure chamber opened, the heart touched with dry filter paper and with the cannula transferred to a small weighing bottle. The time taken from the first putting on of pressure until the heart was again beating on a fresh supply of solution was about 4 min.

A series of weighings under Ringer gave the following results for the heart without cannula

Time	11^{03}	11^{08}	11^{15}	11^{21}	11^{27}	11^{33}	11^{39}	11^{44}
mg	213	209	209	209	205	205	205	205

Now saccharose Ringer (SR) was substituted and the weight began to decrease

Time . .	11^{55}	12^{33}	13^{44}	14^{11}
mg	197	192	189	188

During this time kations were given off to the solutions. The SR showed a concentration of 34.3 mM, the samples at 12^{33} and 13^{44} showed 34.7 and 35.0 respectively, while the last sample at 14^{11} showed only 33.8. During the first SR periods the heart beat was regular, but the amplitude very low (0.05/60), after 20 min. it became slow and irregular, but remained low, and later again the slow contractions appeared in groups with pauses of 2 to 3 minutes.

After the SR the heart was given normal R. The pulse became regular at once (0.25/60) and the weight increased

Time . .	14^{18}	14^{24}	14^{31}	15^{23}
mg	192	193	198	200

At 14^{22} and 14^{29} samples were taken and analysed for saccharose. If SR filled only the extracellular dead space, overestimated at 100 μl, and became completely mixed with the 2 ml R added the first sample should contain 142 mg %; the second 7 mg and the third 0.35. Instead of that the second sample showed 47.5 mg % and the third 29.7, which is possible only when some saccharose was eliminated also from the cells, as we know from the experiments described in the preceding paper that a complete mixing of the fluid in the extracellular space is attained in a heart beating at 0.23/60 in a minute or less. We must conclude therefore that some saccharose entered the muscle cells, although no equilibrium was attained.

These experiments ought to be repeated and extended, but as it appears unlikely that we shall get an opportunity to do so we feel justified in publishing them in this fragmentary state.

Summary.

In a frog heart working on a solution in which 88 mM of Na and Cl are replaced with 175 mM glucose the sodium in the heart muscle cells is largely replaced by glucose. This replacement is reversible when the heart is put back into normal Ringer.

Even saccharose can penetrate slowly into the muscle cells and replace the sodium.

Inconclusive evidence (a single experiment) is presented indicating that the sodium within the cells can be reduced below the outside concentration.

References.

KROGH, A., A-L. LINDBERG and B. SCHMIDT-NIELSEN, Acta Physiol. Scand. 1944, *7*, 221.
SAKAI, T., Z. Biol. 1913, *62*, 295.

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Further Investigations on the Effect of Tyrosine and Related Substances on the Ripening of the Reticulocytes.

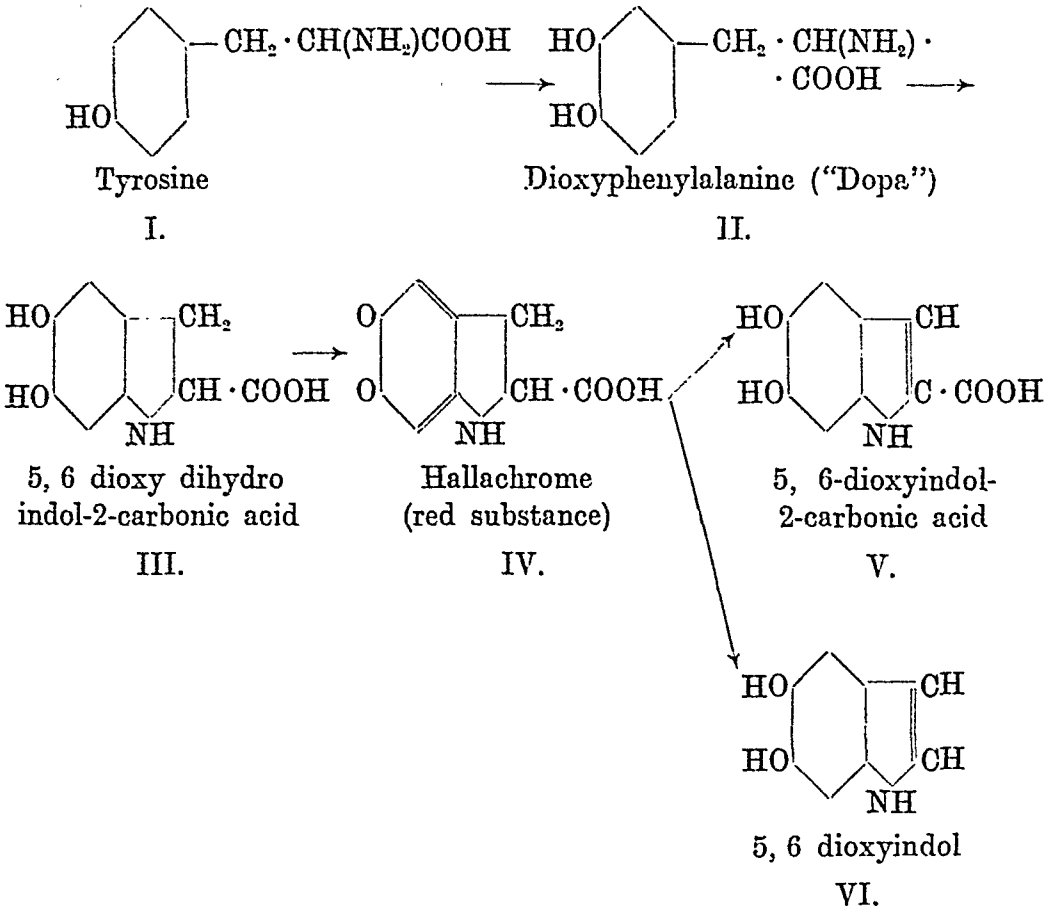
By

INGER GAD, ERIK JACOBSEN and CLAUS MUNK PLUM.

Received 27 January 1944.

PLUM (1942) has shown that the ripening of reticulocytes is induced by some principle found in plasma, liver extract and extracts from other organs and tissues. The ripening priniciple consists of at least two fractions, one thermolabile and one thermostable. In liver extract the latter has been identified as tyrosine (JACOBSEN and PLUM 1942). In a subsequent paper (JACOBSEN and PLUM 1942) it was shown that thyrosine in the ripening complex can be substituted by some chemically related substances as tyramine, adrenaline, oxedrine (sympatol) etc., all having a ripening effect in combination with the thermolabile fraction. Other substances as phenylalanine, o- and m-tyrosine have no effect at all. It was then assumed that the tyrosine may be transformed into some indoline-derivative during the action with the reticular substance, a reaction similar to that which happens when tyrosine is transformed into melanin, but at the time we had no further evidence to sustain this hypothesis. In the present paper we report some experiments all showing that some of the mentioned indoline-derivatives have a much greater effect than the original amino acids or amines. As we have found enzymes in the red blood cells increasing the activity of tyrosine, we do not find it improbable to assume that the effect of tyrosine etc. depends on a transformation into similar substances which react with the reticular substance or otherwise interact in the processes of its disappearance.

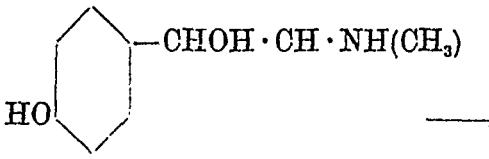
The investigation of RAPER (1932) has shown the following chemical reactions of tyrosine under the influence of tyrosinase:



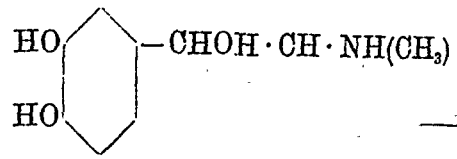
A. Tyrosine-Dopa-Hallachrome.

Similar reactions are seen when adrenaline and oxedrine are broken down. In this case adrenochrome (VII), which corresponds to hallachrome (IV), is formed. From other amines similar substances can be formed, as VIII from βp-oxyphenylpropylmethylamine and IX from tyramine.

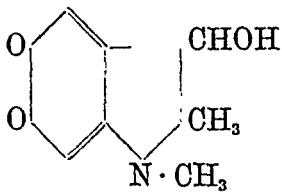
From the reaction chain beginning with tyrosine, we have prepared dopa (II), hallachrome (IV), V and VI, and compared the ripening effect of these substances with that of tyrosine. In a similar manner we have compared adrenochrome (VII) with adrenaline and oxedrine, VIII with βp-oxy-phenylisopropylmethylamine and IX with tyramine.



Oxedrine

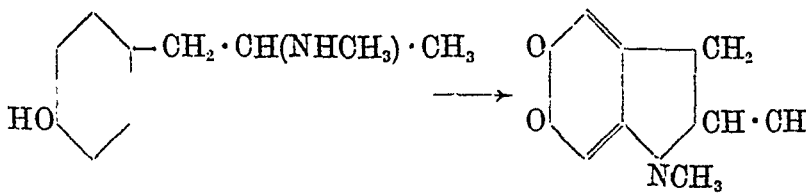


Adrenaline



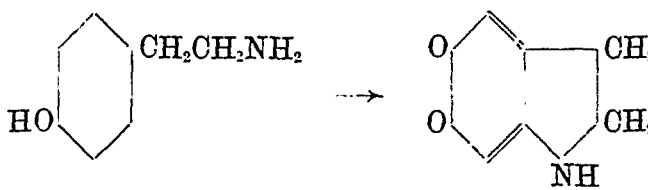
Adrenochrome VII

B. Oxedrine-Adrenaline-Adrenochrome



VIII.

C. βp-oxyphenylpropylmethylamine.



IX.

D. Tyramine

Part I.

Effect of Indolinederivatives. Experimental.

The activity of the substances and solutions was tested in the following way. Red blood corpuscles with about 200 p. m. reticulocytes obtained from anemic rabbits were suspended in a solution containing the thermolabile factor of the ripening principle; to this was added

the tyrosine derivative to be examined. In these experiments we used as thermolabile factor saline extracts from dried hog stomach which PLUM has found very rich in this factor and containing but little of the thermostable factor. The ripening of the reticulocytes was determined as usual [PLUM (1942)] and the monomolecular constant was used as a measure for the ripening rate. The effect of all substances and solutions was compared with that of tyrosine.

A. The Tyrosine-Dopa-Hallachrome Chain.

dl-dioxyphenylalanine (dopa) (II) was prepared according to TAKOOKI SASAKI (1921) from vanilline.[1]

The activity of dopa (II) is found to be about twice that of tyrosine.

Table 1.

Effect of Tyrosine and Dopa.

Reticulocytes incubated in	Monomolecular constants of ripening rates			
	found	corrected for the effect of gastric extract	calculated effect of 1 mg % tyrosine or dopa	
Saline	0.0182	—	—	
Gastric extract	0.0139	0.000	—	
Gastric extract with 10 mg % tyrosine	0.0335	0.0196	0.00196	average 0.00188
Gastric extract with 5 mg % tyrosine	0.0229	0.0090	0.00180	
Gastric extract with 10 mg % dopa	0.0530	0.0391	0.00391	average 0.00395
Gastric extract with 5 mg % dopa	0.0338	0.0199	0.00398	

$$\frac{\text{Effect of dopa}}{\text{Effect of tyrosine}} = 2.10$$

An example is given in table 1. Here the effect of dopa is found 2.10 times that of tyrosine; in six similar experiments this figure was found ranging between 1.92 and 2.22 with an average of *2.06*.

A solution of hallachrome, the red substance of RAPER (1932) was made by the action of enzymes from tyrosine or from dopa: to 1—2 litres 1 p. m. tyrosine was added $^1/_{10}$ volume solution of tyrosinase prepared from mealworms (tenebrio molitor) exactly following the description of HAMMERICH (1931). The solution was adjusted to pH

[1] All chemical preparations were made by I. G.

6.5 with a little acetic acid and kept at 30°. During the incubation the solution was frequently aereated with oxygen. After two hours the enzyme was precipitated by addition of 20 cc 1 p. c. acetic acid per 1.5 litre and centrifuged off. The clear claret coloured solution contains mainly unaltered tyrosine mixed with hallachrome and perhaps some other oxydation products of tyrosine. A small part was tested in the ripening experiments, on another small part the unaltered tyrosine was determined after the method of HAMMERICH (1931). The rest was divided into three parts. Part one was treated with phenylhydrazine as described by VEER (1939), and the phenylhydrazone of the hallachrome was crystallized out; the precipitate is dissolved in hot alcohol and brought to crystallisation by addition of water and cooling. The phenylhydrazone was identified through its nitrogen content and its melting point. The second part of the hallachrome solution was kept under vacuum in 18—24 hours; then the red colour had disappeared, the hallachrome being transformed into dioxyindol (V) (RAPER 1932); the third part was treated with sulphur dioxide in 18—24 hours. In this case the red colour disappears also. The excess of sulphur dioxide was removed in vacuum. According to RAPER (1932) the hallachrome is then transformed into dioxyindolcarbonic acid (VI). Attempts to isolate and identify the two indol derivatives were in vain, but both solutions gave a very marked qualitative reaction for indol when tested with dimethylaminobenzaldehyde.

As to the affect of hallachrome figs. 1 and 2 give the results of some of the experiments. In fig. 1 a solution of 1 ‰ tyrosine treated with a tyrosinase preparation was tested against pure tyrosine. From three different concentrations of tyrosine tested (5, 10 and 20 mg %) it was in a manner similar to that shown in table 1 calculated that the effect of 1 mg % tyrosine was 0.00233. The tyrosinase-treated solution contained 77.2 % unaltered tyrosine of the total dry matter and hence it is possible to calculate the fraction of the ripening constant found which is caused by the tyrosine; this is only about 2 per cent of the total. The rest of the dry matter in the tyrosinase treated solution is oxydation products of tyrosine, mainly *hallachrome*. If we reckon the whole rest to be hallachrome, we can calculate the *minimal* effect of hallachrome, as the further oxydation steps have no effect. This effect is found to be an average of 0.103 per milligram per cent or at least 45 times that of tyrosine. The results are given in fig. 1.

In the experiments given in fig. 2, we have calculated the *maximal* effect of hallachrome. A 1 p. m. solution of dopa was treated with tyrosinase in the manner described; from the amount of phenylhydrazone isolated it could be calculated that at least 26.3 per cent of the dry matter was hallachrome. If the 73.7

per cent is presumed to be unaltered dopa it is possible in a similar way as mentioned above to calculate the part played by the hallachrome in the ripening constant found. Since it is probable that not all the hallachrome is isolated as phenylhydrazone, this figure must represent the maximal effect. This is found to be 0.262 per milligram per cent hallachrome against 0.00247 per milligram per cent tyrosine in the same experiment. The effect of hallachrome is thus not more than 106 times that of tyrosine. As hallachrome cannot be isolated in pure form it is impossible to give the exact relation between the effect of tyrosine and hallachrome, but it must be placed between 45 and 105.

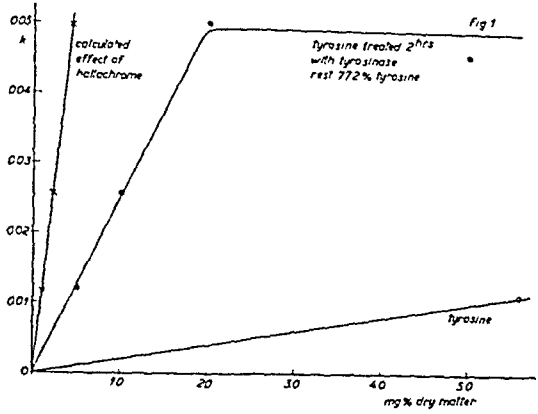


Fig. 1. Effect of tyrosine on reticulocyte ripening before and after treatment with tyrosinase. (Ordinate: ripening constant.)

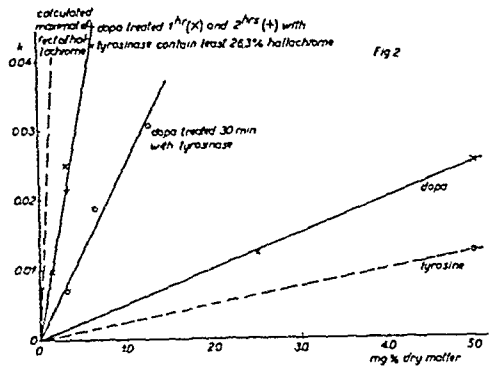


Fig. 2. Effect of dopa on reticulocyte ripening before and after treatment with tyrosinase.

The hallachrome solution did not have any ripening effect by itself but only in combination with the thermolabile factor.

After being kept under vacuum during 18—24 hours or treated with sulphurdioxide the hallachrome solution loses its ripening effect. Thus neither dioxyindolcarbonic acid (V) or dioxyindol (VI) have any ripening effect.

B. The Oxedrine-Adrenaline-Adrenochrome Chain.

Adrenochrome was prepared in a similar way as hallachrome. 1,000 cc of a solution of oxedrine tartrate containing 1 p. m. oxedrine base was treated with 100 cc tyrosinase preparation under oxygen; small portions were taken out after $^1/_2$, 1 and 3 hours incubation at 30°.

After 3 hours acetic acid was added to the whole portion in order to precipitate the enzyme which was centrifuged off. The rest was treated with phenylhydrazine as described by VEER (1939). From the 1,100 cc solution was isolated 200 mg phenylhydrazone, corresponding to a content of about 10 mg per cent adrenochrome in the solution. In the samples taken out were found 0.91 p. m. dry matter of which 91.7 per cent and 87.7 per cent after $^1/_2$ and 3 hours were found to be oxedrine. Thus the solution after 3 hours incubation contained 91 mg per cent dry matter of which 80 mg per cent was unaltered oxedrine and 10 mg per cent could be isolated as adrenochrome, it must also be presumed that all oxedrine disappeared is converted into adrenochrome.

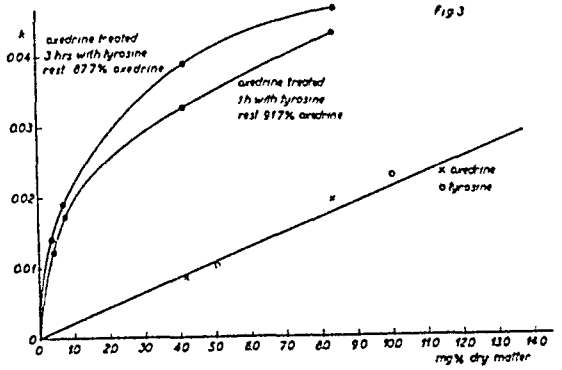


Fig. 3. Effect of oxedrine (sympatol) on reticulocyte ripening before and after treatment with tyrosinase.

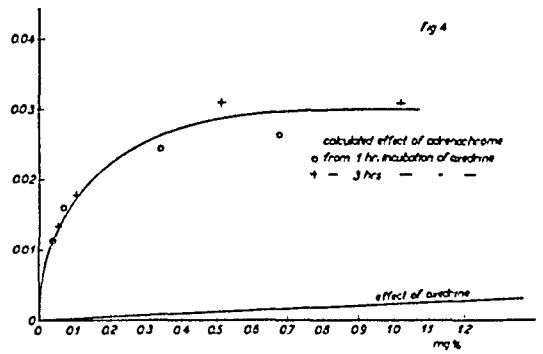


Fig. 4. Calculated effect of adrenochrome on reticulocyte ripening.

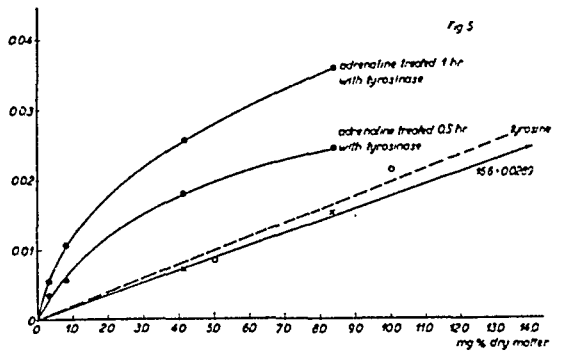


Fig. 5. Effect of adrenaline on reticulocyte ripening before and after treatment with tyrosinase.

From fig. 3 it can be seen that the effect of oxedrine is very much increased after incubation with tyrosinase. From the known effect of oxedrine and from the constant of oxedrine in the solution we have calculated the effect of adrenochrome which is given in fig. 4.

The effect of tyrosinase treatment on adrenaline is given in fig. 5; here we made no determination of non altered adrenaline in the solution, and as the

isolation of adrenochrome-phenylhydrazine only gave a very poor yield, it is impossible to calculate the isolated effect of adrenochrome from adrenaline.

C. βp-Oxyphenylisopropyl-Methylamine — VIII.

1½ litre 1 ‰ solution of the base was treated in the usual way with 150 cc tyrosinase preparation during 3 hours at 30°. After the incubation 89.6 % amine was refound and 400 mg substance was precipitated with phenylhydrazine. If the phenylhydrazon was pure it would correspond to 248 mg VIII or 16.7 per cent. Thus the major part of the amine lost must probably be present as the indoline derivative. In fig. 6 is given the result of an experiment and in fig. 7 the calculated effect of VIII compared with that of βp-oxyphenylisopropylmethylamine.

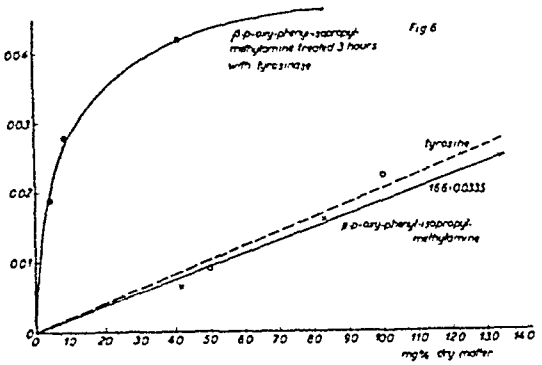


Fig. 6. Effect of βp-oxy-phenylisopropylamine on reticulocyte ripening before and after treatment with tyrosinase.

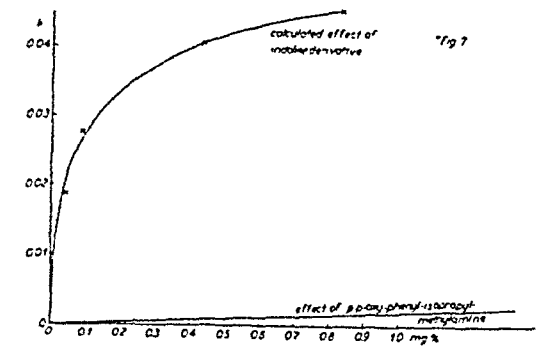


Fig. 7. Calculated effect of indoline derivative of p-oxy-phenylisopropylamine on reticulocyte ripening.

D. Tyramine — IX.

A solution of tyramine hydrochloride corresponding to 1 p. m. tyramine was treated with tyrosinase as described and the preparations tested against the reticulocytes. After 2 hours incubation with the enzyme was 63.6 p. c. and after 3½ hours 61.0 p. c. of the tyra-

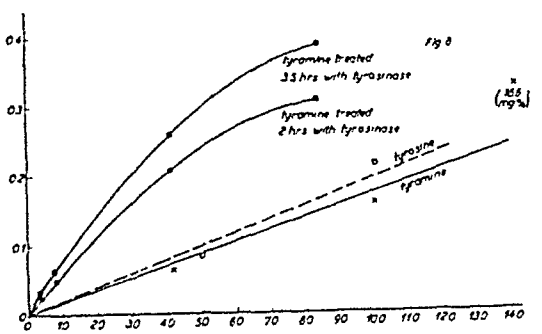


Fig. 8. Effect of tyramine on reticulocyte ripening before and after treatment with tyrosinase.

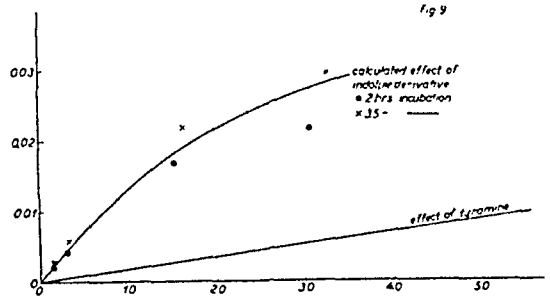


Fig. 9. Calculated effect of indoline derivative of tyramine on reticulocyte ripening.

mine found unaltered. Attempts to isolate the indoline derivative as phenylhydrazone gave only a very poor yield as the precipitate was too small to become recrystallised. The figs. 8 and 9 show the results plotted in a similar way as the other experiments.

E. Diiodotyrosine.

This substance is not attacked by tyrosinase preparations hitherto used. This corresponds well to the fact that the effect of diiodotyrosine is not increased after incubation with the mealworm extract. The results of an experiment are given in the following table 2.

Table 2.

Reticulocytes incubated in	Monomolecular constants of ripening rates	
	found	Corrected for the effect of gastric extract
Saline	0.0115	—
Gastric extract	0.0128	—
Gastric extract with 10 mg % tyrosine	0.0225	0.0097
Gastric extract with 20 mg % Diiodotyrosine	0.0313	0.0185
Gastric extract with 10 mg % Diiodotyrosine	0.0205	0.0077
Gastric extract with 20 mg % Diiodotyrosine treated with tyrosinase during 2 hours	0.0308	0.0180
Gastric extract with 10 mg % Diiodotyrosine treated with tyrosinase during 2 hours	0.0194	0.0066

Discussion of Part I.

The principal result of the experiments is that an incubation with tyrosinase increases the ripening effect of an amino acid or an amine. This happens only when the amine is able to form

an indolinederivative. Substances like diiodotyrosine which are not attacked by the tyrosinasepreparation used do not alter their ripening effect. The preparations with the strongest ripening effect contain mainly the red indoline-derivative and unaltered amine, and it is highly probable that the increased effect is due to the former.

So far it is not *proved* that the increased activity is due to the formation of the indoline derivative as it is impossible to isolate these quinones in pure form and the quinones are destroyed in attempts to hydrolyse the phenylhydrazone with diluted acid. It is, however, very improbable that the increased effect should be caused by some other substance present in minor quantities. In the case of oxedrine and βp-oxyphenylisopropylmethylamine practically all the amine lost is found again as indoline derivative, and if some other substance was responsible for the increase in effect, this substance must possess an effect several thousand times that of the original amine which seems most improbable.

From the determination of unaltered amine and the weight of the isolated phenylhydrazone of the indoline-quinone it can be calculated that the indoline derivative has an effect of 50 to more than 100 times that of the original amine.

An exception is formed by tyramine, where the indoline derivative apparently has only ten times the effect of tyramine. This is probably due to the fact that only a small part of the tyramine lost is present in the solution as indoline derivative, the rest being transformed into ineffective substances; the fact that only very little phenylhydrazone could be isolated from the solution supports this assumption.

Thus while tyrosine is effective in a concentration of 1 : 10,000, hallachrome yields the same effect in concentrations between 1 : 500,000 and 1 : 1,000,000. This great increase gives the idea that the effect of tyrosine on the reticulocytes is based on a transformation into hallachrome in the blood corpuscles. In a similar way oxedrine and adrenaline should be transformed in adrenochrome and the other effective amines into the corresponding derivatives. Further alterations of the hallachrome and the similar substances give no effect at all.

That dopa has twice the effect of tyrosine gives some support to the hypothesis; on the other hand adrenaline has approximately the same effect as oxedrine where an increased effect should be expected. Against the hypothesis of a necessary transformation

into indoline derivatives speaks the fact that diiodotyrosine has a ripening effect in spite of its inability to form an indoline derivative directly, but the blood corpuscles may be able to split off the iodine and thus make it possible to form the substance wanted.

The whole hypothesis, however, depends on whether the blood corpuscles are able to form the indoline derivative; in the second part of this paper it will be shown that tyrosine is decomposed by the red blood corpuscles. Furthermore it is possible to isolate from the blood corpuscles an enzyme or an enzyme complex with an activating effect on tyrosine equal to the effect obtained by tyrosinase preparations.

Part II.

Influence of Erythrocytes on Tyrosine.

The blood corpuscles from 260 cc blood from an anemised rabbit were separated from the plasma in the centrifuge, washed and divided in two portions. The one was added 50 cc saline and remained as a blank while the other was added 50 cc saline with 10 cg tyrosine. Both were kept at 37° and aereated with oxygen. The tyrosine was estimated at once and after 2, 4 and 6 hours. The results are given in table 3.

Table 3.

Influence of red blood corpuscles on tyrosine			
	Found mg % "tyrosine"		
	in blank	in suspension added tyrosine	in suspension added tyrosine — blank
at the beginning of the incubation	9.1	*51.6*	42.5
after 2 hours at 37°	9.8	52.1	42.3
after 4 hours at 37°	9.8	49.8	40.0
after 6 hours at 37°	11.1	50.5	39.4

A decrease of about 3 mg% is observed. The major part of this decrease, however, is due to an increase of "tyrosine" in the blanks, presumably phenol bodies liberated during the incubation.

As the decrease in tyrosine is so much smaller than the "tyrosine" found in the blank, we dont regard experiments of this kind quite reliable. Hence we have made supplementary experiments in order to examine whether tyrosine or tyrosine-like substances are decomposed by the red blood corpuscles. PLUM (1943) has shown that the amount of ripening substances in plasma is considerably reduced when whole blood is stored. The reduction will be larger when the temperature is increased. Stored under the same conditions plasma showed comparatively little decrease in the quantity of the ripening substances. Earlier we have shown [JACOBSEN and PLUM (1943)] that the ripening principle in plasma consists of two fractions, one of which can be replaced by tyrosine. Unlike from liver extracts tyrosine cannot be isolated from plasma, but the thermostable part of the ripening principle in plasma must nevertheless be closely related to tyrosine. Not only can it be replaced by tyrosine, but injections of tyrosine to an animal gives a slight increase in the ripening principle of its plasma [PLUM and PLUM (1943)]. Now we can show that the decrease in ripening principle after storing is due to a decrease in the "tyrosine"-factor which is destroyed by the blood corpuscles. In table 4 an experiment showing this is given.

Table 4.

Blood from	Whole blood stored at room temperature in	Ripening index of plasma	
		without addition of tyrosine	with 0.1 ‰ tyrosine
Normal rabbit (15 ‰ reticulocytes)	0 hours	0.69	0.70
	3 »	0.63	0.68
	6 »	0.58	0.66
Anemised rabbit (375 ‰ reticulocytes)	0 hours	0.94	0.95
	3 »	0.85	0.95
	6 »	0.75	0.91

We have to assume that the products formed from the tyrosine lost remain inside the corpuscles.

Finally we can show that addition of tyrosine and dopa is able to increase the oxygen consumption of the blood corpuscles. This should be expected if tyrosine is transformed into dopa and dopa into hallachrome by the erythrocytes. As seen by fig.

10 this increase is not a considerable one, but the result was reproduced several times.

Taken as a whole there must be little or no doubt that tyrosine and dopa can be affected by the erythrocytes and moreover it is rather probable that the breakdown is an oxydation, but these experiments give no evidence that hallachrome is formed. We therefore attempted to make a "tyrosinase"-preparation from blood corpuscles and test its effect against tyrosine and dopa. When treated with this enzyme preparation, solutions of tyrosine and specially of dopa turned black as seen with other tyrosinases but we did not succeed in isolating the phenylhydrazone of hallachrome in our experiments with extract from blood corpuscles. We could, however, show that tyrosine and dopa were more active after treatment with the enzyme preparation from erythrocytes.

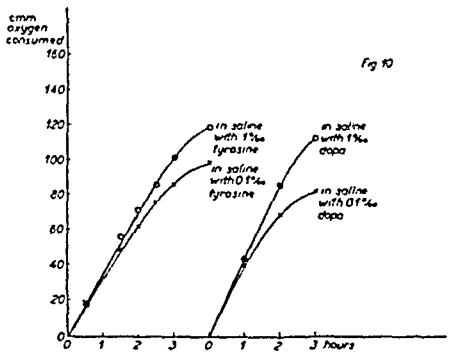


Fig. 10. Effect of tyrosine and dopa on the respiration of red blood corpuscles.

The enzyme solution was prepared in the following way. (The procedure described is only a preliminary example. Further studies on the nature of this enzyme are in progress and will be published by GAD.) Blood from normal or anemised rabbits is centrifuged and the erythrocytes hemolysed through addition of distilled water. When the "shadows" are centrifuged off acetic acid is added to a final concentration of about m/50. The solution is filtered and the enzyme then adsorbed on purified kaolin which is washed several times with m/50 acetic acid and eluted with m/50 ammonia.

The solution which at times can be free from hemoglobin, is used as enzyme preparation. A solution of 1 ‰ tyrosine or dopa was incubated at 37° with a tenth volume of the enzyme preparation as described with the tyrosinase preparation from meal-worms. The tyro-

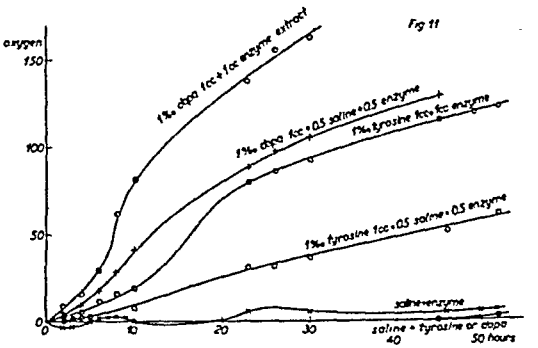


Fig. 11. Oxygen consumption of tyrosine and dopa in presence of enzyme prepared from red blood corpuscles.

sine and dopa solutions were then tested against reticylocytes in presence of gastric extract. The results from a typical experiment are given in table 5.

Table 5.

Effect of tyrosine and dopa treated with encyme-preparations from erythrocytes (enzyme extract).

6. 6. 43.

Reticulocytes suspended in			Monomolecular constant of ripening rate	Ripening constant
Saline	Gastric extract			
2.0 cc	—	—	0.0129	—
1.9 cc	—	Tyrosine 1 ‰ 0.1 cc . . .	0.0132	0.0003
1.9 cc	—	Dopa 1 ‰ 0.1 cc	0.0133	0.0004
1.9 cc	—	0.1 cc enzyme extract . . .	0.0136	0.0007
0.8 cc	1.7 cc	—	0.0157	0.0028
0.2 cc	1.7 cc	0.1 cc enzyme extract . . .	0.0161	0.0033
0.2 cc	1.7 cc	0.1 cc 1 ‰ Tyrosine . . .	0.0304	0.0175
0.1 cc	1.7 cc	0.1 cc 1 ‰ Tyrosine . . 0.1 cc enzyme extract . .	0.0378	0.0249
0.1 cc	1.7 cc	0.1 cc 1 ‰ Tyrosine . . 0.1 cc enzyme extract . . incubated 2 hours at 37°. before ripening experiment	0.0481	0.0352
0.1 cc	1.7 cc	0.1 cc 1 ‰ Tyrosine . . 0.1 cc enzyme extract . . incubated 4 hours	0.0527	0.0398
0.2 cc	1.7 cc	0.1 cc 1 ‰ Dopa	0.0464	0.0335
0.1 cc	1.7 cc	0.1 cc Dopa 1 ‰ . . . . 0.1 cc enzyme extract . .	0.0556	0.0427
0.1 cc	1.7 cc	0.1 cc Dopa 1 ‰ . . . . 0.1 cc enzyme extract . . incubated 2 hours	0.0734	0.0605
0.1 cc	1.7 cc	0.1 cc 1 ‰ Dopa . . . . 0.1 cc enzyme extract . . incubated 4 hours	0.0802	0.0673

It is seen that the effect of both tyrosine and dopa has undergone a marked increase in effect after treatment with the enzyme preparation from blood cells. The increase it not so great as seen after treatment with tyrosinase from meal-worms, but the concentration of the enzyme is much lower. Fig. 11 shows the oxygen uptake of tyrosine and dopa under influence of the enzyme preparation, and it is seen that only very little oxygen is consumed

during the first hours, but a calculation shows that enough oxygen is consumed to form an amount of hallachrome necessary to explain the increase in activity found.

Discussion.

The experimental evidence shows that the red blood corpuscles possess enzymes able to convert tyrosine and dopa into more active substances. The results of the experiments with mealworm tyrosinase where hallachrome was isolated, combined with the fact that tyrosine treated with enzyme from blood corpuscles gives an oxygen uptake, makes it probable that hallachrome is formed under the influence of the erythrocytes. So far nothing speaks against the assumption that hallachrome is the substance effective in the reticulocyte ripening and that the effect of tyrosine and dopa should depend on a transformation of these substances into hallachrome in the blood corpuscles.

The amount of the enzyme in the blood corpuscles is, however, rather limited and the question arises if it is large enough to get a reasonable amount of tyrosine converted. According to table 5 0.1 cc enzyme extract incubated with 0.1 cc 1 p. m. tyrosine is sufficient to double the activity of the tyrosine solution in two hours. Under the assumption that this increase is due to the formation of hallachrome and that hallachrome has an effect of 100 times that of tyrosine, 1 per cent of the tyrosine should be transformed into hallachrome by the enzyme during the two hours. As the enzyme extract is prepared 1 cc extract corresponds to 5 cc original blood; the blood corpuscles of 0.5 cc anemic blood therefore contain at least sufficient enzyme to make a hallachrome solution of 1 : 200,000 during 2 hours from a solution of tyrosine 1 : 2,000.

20 per cent of the anemic blood is erythrocytes. The enzyme present in 0.1 cc extract is thus found in the water-phase of 0.1 cc blood corpuscles, i. e. in 0.05 cc. According to this calculation the concentration of the enzyme in the water-phase of the blood corpuscles is 4 times that of the solution incubated. If the activity of the enzyme is proportional to the concentration, this means that the amount of enzyme in the blood corpuscles is sufficient to make a hallachrome solution of 1 : 50,000 out of a tyrosine solution of 1 : 2,000 in two hours. As the ripening effect is proportional to the tyrosine concentration, it is logical to assume

that the effect of blood cell tyrosinase is proportional to the substrate concentration as well. Now in our ripening experiments we generally use a tyrosine concentration of 1 : 10,000. The enzyme concentration should be sufficient to make from this solution a hallachrome concentration of 1 : 250,000 in two hours, which is four times as much as equivalent to a tyrosine concentration of 1 : 10,000. When it is considered further that the preparation of enzyme extract must be accompanied by considerable loss, it is seen that the amount of enzyme in the blood corpuscles is sufficient and does not speak against the assumption that tyrosine in the reticulocytes must be converted into hallachrome before it can act in the ripening processes.

It may, however, be possible that the active tyrosine derivative formed by enzyme from blood corpuscles is not identical with hallachrome. Until we have succeeded in isolating hallachrome from incubation experiments with tyrosine or dopa and blood enzyme, the explanation given above must remain hypothetical.

Summary.

By means of tyrosinase from meal-worms, indoline derivatives are formed from tyrosine, adrenaline and other related substances.

It is shown that the effect on reticulocyte ripening of tyrosine, adrenaline, βp-oxyphenylisopropylamine and tyramine is only 10 to 1 % of the effect of the corresponding indoline derivatives: hallachrome, adrenochrome etc. Dioxyindole and dioxyindole-carbonic acid have no ripening effect.

Tyrosine is decomposed by red blood corpuscles and from red blood corpuscles we have prepared enzymes able to convert tyrosine and dioxyphenylamine into substances more active towards reticulocyte ripening. The same enzyme preparation is able to form melanine from tyrosine and dioxyphenylalanine, and to give rise to the oxygen consumption in solutions of these substances.

This makes it possible that hallachrome can be formed from tyrosine in the erythrocytes and a calculation shows that the enzyme concentration inside the erythrocytes is sufficient to support the assumption that the effect of tyrosine on the reticulocytes is due to its conversion into hallachrome with the corpuscles.

Literature.

HAMMERICH, T., Biochem. J. 1931. *239*. 273.
JACOBSEN, E., and C. MUNK PLUM, Acta Physiol. Scand. 1942. *4*. 272, 278.
—, and C. MUNK PLUM, Ibidem 1943. *5*. 1.
PLUM, C. M., Ibidem 1942. *4*. 259.
—, Ibidem 1943. *5*. 175.
—, and R. PLUM, Ibidem 1943. *5*. 380.
RAPER, H. S., Ergebn. Enzymforsch. 1932. *1*. 270.
SASAKI, T., Ber. Dtsch. Chem. Ges. 1921. *54*. 163.
VEER, W. L. C., Rec. trav. chim. Pays Bas 1939. *58*. 943.

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Investigations on the Microphone Effect of the Cochlea with some Remarks on a New Technique.

By

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Received 30 January 1944.

Despite a close knowledge of the histology of the inner ear and numerous attempts to explain its function it must be admitted that to this day we do not know how the transformation of sound waves into the action potentials of the auditory nerve and the central auditory pathways takes place. The discovery of the so-called WEVER- and BRAY phenomenon in 1930 aroused increased interest in this important problem, and a possibility has been created of getting nearer to its solution by means of experimental work.

During the attempt to lead off electric potentials from the auditory nerve of cats exposed to sound effects WEVER and BRAY found such powerful and pure potentials that by suitable amplification and transference to a loud-speaker the words spoken into the ear of the cat could be distinctly understood. Simple action potentials from the auditory nerve had previously been known, but the potentials observed by WEVER and BRAY differed in essential particulars from the action potentials in the auditory nerve. It was not until the succeeding years, however, that it became clear that the potentials first observed by WEVER and BRAY arose in the cochlea and had nothing to do with the action potentials of the nerve. ADRIAN (1931) introduced the term microphone effect and SAUL and DAVIS pointed out the great differences between the action potentials from the auditory

nerve and the microphone effect of the cochlea (1932). While the action potentials from the auditory nerve are limited to rather low frequencies, are affected by narcotics, and are released from the auditory nerve and the central auditory pathways, the microphone effect of the cochlea includes a much larger area of frequency, is not affected by narcotics, and is released from the cochlea.

A number of works on the WEVER and BRAY phenomenon from the years that followed showed that this phenomenon is not an artefact, but a biological phenomenon associated with the living tissue in the cochlea. On two important points, however, we still lack clarity; despite numerous works on this interesting effect it has not yet been possible to agree as to where in the cochlea the potentials arise, just as it has not yet been settled whether these potentials are a necessary link in the whole complicated mechanism of the perception of sound or only an accidental concomitant without any significance whatever for hearing. As to the point at which the potentials arise, attention has especially been directed towards the hair cells in the organ of Corti and to the Reissner membrane. A change of form in the hair cells caused by currents in the perilymph and endolymph may give rise to electric potentials in the form of the so-called piezo-electric effect; such a change in the form of the cells in the organ of Corti is quite conceivable according to the histological structure (GUILD, 1937). A number of authors have pointed out the probability of the hair cells being the place of origin of the microphone effect. By examining animals with a congenital absence of or a defective organ of Corti DAVIS, DERBYSHIRE, LURIE and SAUL (1933), HUGHSON (1937) and others found that the microphone effect was entirely or partially abolished. Other authors (EYSTER and BAST (1937), HALLPIKE and RAWDON-SMITH (1934)), however, have found that the microphone effect may be present despite absence or degeneration of the organ of Corti; these authors instead ascribe decisive importance to the Reissner membrane for the rise of the potentials. A degeneration of the organ of Corti has been produced by cutting of the auditory nerve or by exposing animals to a somewhat loud noise for some length of time. A degeneration of the organ of Corti together with the spiral ganglion can also be produced by means of ascaridol (the active substance in chenopodium oil), as shown amongst others by OKA (1929). So as to throw more light on these questions we have tried

by means of injection or ingestion by mouth of ascaridol into guinea-pigs to produce such an elective degeneration of the organ of Corti; the succeeding investigations on the WEVER and BRAY effect combined with histological investigations may possibly clear up the bearing of the hair cells on the microphone effect. These experiments have not yet been terminated.

While working out the technique for the quantitative estimation of the microphone effect of the cochlea on guinea-pigs we have amongst other things ascertained whether this effect could be influenced by various drugs and in the first place salicylic acid and quinine. Audiometer investigations made by us on human subjects (1943) suggest that the point of attack of the salicylic acid is either in the organ of Corti or more probably centrally to it (in contrast with the findings of FALBE-HANSEN (1941)). If the point of attack is the organ of Corti and the microphone effect is released there, it would be natural to suppose that the microphone effect could be influenced by salicylic acid.

Technique.

In a single case the investigations on the microphone effect were made on a cat, but in all other cases on guinea-pigs. Before the animal is anesthetised it is ascertained whether Preyer's reflexes are present. Urethane anesthesia is used. The animal is placed on its back on a warm operating table. In order to avoid compression of the trachea a tracheal cannula is introduced, after which the muscles are pushed aside with a chiefly blunt instrument, keeping close to the inner side of the mandible and m. pterygoideus int. (it is of great assistance to the beginner to have the skull of a guinea-pig to look at). As a rule the bulla can be exposed in a few minutes practically without bleeding. The space is very limited, still one can manage very well without any assistant as long as one has a frontal mirror and a small deep-going blunt self-retaining retractor at one's disposal. The muscles covering the bulla deep down are pushed aside with a cotton wad, no sharp instruments should be used here as this may cause troublesome bleeding. The bulla as a rule quite thin may be opened with a knife, and when the membrane extended in the bulla has been removed the cochlea is freely accessible to an electrode which is placed directly on the apex.

A chloridised silver electrode is applied, about 7 cm long and 1 mm thick. *It is of vital importance to use electrodes freshly chloridised immediately before each experiment.* The chloridisation is carried out as follows: the silver wire is well cleaned and entirely freed from fat after which it is treated with a saturated solution of sodium thiosulphate so as to remove all silver chloride; this is of course of special importance if the electrode has previously been chloridised. The quite bright silver

wire is boiled in water, after which the chloridisation takes place in 1/10 n HCl, the silver wire being connected to the positive pole of a 2 volt accumulator; a silver plate is used as cathode. Electrolysis for 10 minutes with 3—4 mA; now and then the current is transiently increased to 10 mA. Avoiding contact with the chloridised part of the electrode the latter is covered with an isolation cuff, as generally used in radio technique. At least two electrodes were always made for each experiment, so that the electrode may quickly be changed if by rubbing against the cochlea it should lose some of the silver chloride, in which case irregular potentials will arise. The electrode is placed with the point directly in contact with the apex of the cochlea without otherwise touching the animal. It is important that there should be no moisture on the cochlea which must therefore, if required, be carefully wiped with hydrophilous cotton wool immediately before the electrode is applied. As the anode was used a spring terminal which was fixed in the shaved and scarified auricle which had been scraped with sandpaper, so that the distance between the cathode and the anode was very short.

The experimental animal was placed on a grounded copper plate, in some of the experiments in a grounded Faraday cage, in other experiments without this cage ("floating"); equally good results are obtained with and without the Faraday cage. As a source of sound we used for the quantitative measurements a tone generator (*radiometer* type HO 1 D) which gives sinusoidal oscillations from 20 to 20,000 cycles. The tone-generator is connected over an attenuator (*radiometer* LF, type LP 3, to a crystal telephone (*automatic*), built into a grounded metal screen. Before the microphone of the telephone was placed a 2 cm long 5 mm wide metal tube and connected to this again a 3 cm long rubber tube with the same lumen; this rubber tube was adjusted to the auditory canal of the experimental animal. The cathode was carried over an alternating current intensifier a. m. BUCHTHAL-NIELSEN to a cathode ray oscillograph (*radiometer* type OSG 15) or a loudspeaker which could be placed in a room lying far from the room where the experiment took place so that the currents led off from the cochlea could be seen or heard at will. To make sure that the potentials observed were due to the microphone effect and not merely induced electrical phenomena the rubber tube leading to the auditory canal was compressed: in this way the potentials produced by the microphone effect will entirely disappear.

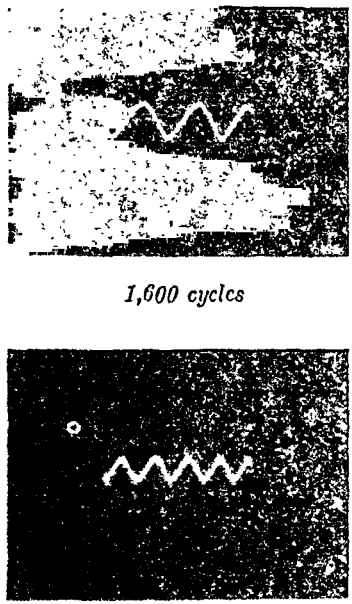
The above technique, at which we have only arrived after a long series of preliminary experiments, has been so carefully described because it seems to give essentially better results than devices previously described in the literature.

Experimental Results.

In all cases with a positive Preyer reflex we found a very considerable microphone effect. The intensity of the potentials might rise to 1 millivolt (the literature as a rule only gives values of up to

100 microvolts (BRAY and LAWRENCE (1941)). Fig. 1 shows a photograph of the oscillations at a frequency of 1,600 and 3,200 cycles.

The intensity of the potentials attained appears clearly from the fact that from any part of the 8 × 4 m large laboratory in which the guinea-pig was placed even ordinary talk could be distinctly understood through the loud-speaker, which of course in these experiments was placed in another part of the building; it was even possible to recognise the speaker by the voice from the loud-speaker, though it had the characteristic metallic ring. With the experimental arrangement we used it was possible both to see and to hear a distinct microphone effect from the cochlea in guinea-pigs *within a frequency field of from about 20—30 cycles right up to 14,500 cycles. This is essentially higher than that previously found for guinea-pigs* (cf. EYSTER, BAST and KRASNO (1937) who found the maximal frequency to be about 4,000 cycles). On cats microphone effects have been observed of up to 16,000 cycles (DAVIS, DWORKIN et al. 1932). The microphone effect may, however, be present at both higher and lower frequencies; but with the technique employed by us it was not possible to demonstrate frequencies outside the field mentioned, which corresponds approximately to the whole of the frequency range audible to man. By subduing the tone led to the ear of the guinea pig to just visible potentials (c. 0.01 mV) on the cathode ray oscillograph for the different frequencies by a suitable intensification (c. 100,000) we found the following average "electric audiogram" for guinea-pigs (Fig. 2):



1,600 cycles

3,200 cycles

Fig. 1. Potentials led off from the cochlea photographed on the cathode ray oscillograph — 1,600 and 3,200 cycles.

As previously pointed out in the literature, this audiogram is in good agreement with the audiogram known from man. It should be noted that in all the experiments the leads were from the apex of the cochlea. On leading off from other parts of the cochlea we found essentially lower potentials.

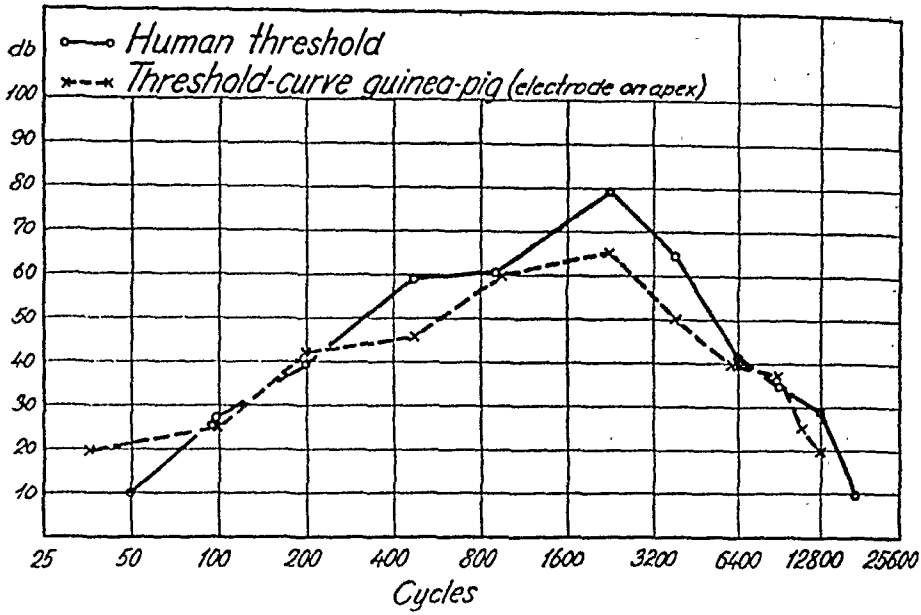


Fig. 2. Audiogram taken from human subjects and an "electric audiogram" taken from guinea pigs with the same telephone.

In all our experiments the microphone effect kept practically unchanged for several hours. Only at the incidence of death which was brought about by intravenous injection of a couple of millilitres of a 10 % KCl solution or by bleeding (cutting of the heart or of the femoral artery), did we notice a quite sudden fall in the potentials to about 1/5 of the original intensity, after which we observed a gradual and slow decrease in the potentials in the course of the next hours as found also by DAVIS, DERBYSHIRE, LURIE and SAUL (1933).

By an accidental destruction of the apex of the chochlea on applying the electrode we have seen in a couple of the experiments that the microphone effect for the deep tones (100—1,500 cycles) decreased or entirely disappeared while for the high frequencies (2,000—14,000 cycles) it remained unchanged. This agrees with the generally recognised frequency localisation to the cochlea according to which the deep tones are localised to the apex while tones of high frequency are localised to the base.

In a number of cases we found *guinea-pigs in which Preyer's reflex was absent;* these animals showed inflammatory changes in the middle ear with pus in the bulla, which was generally very thick-walled. We have never been able to register a microphone effect from such ears with abolished Preyer's reflex; this reflex

is tried for each ear separately. A high tone (c. 2—3,000 cycles) is sounded loudly close to the ear to be tested. Histological investigations of the inner ear were not made in these cases; but I should think that there must have been considerable inflammatory changes in the cochlea.

As already mentioned, a collection of fluid in the bulla around the electrode greatly influences the potentials which decrease in size, so that in some of the experiments it was necessary now and then to dry the bulla with a little hydrophilous cotton. A few drops of procaine dripped into the bulla (½ %) at once abolishes the potentials; after drying, the potentials appear again at their original intensity; this effect, however, is not due to the procaine but only to a short-circuiting through the fluid, the same phenomenon appearing when a couple of drops of physiological saline solution are dripped into the bulla (cf. EYSTER et al. (1937)).

As already mentioned, urethane anesthesia was used in our experiments. These showed that the microphone effect is unaffected even by deep urethane anesthesia, just as previous investigations have shown that ether anesthesia does not affect the phenomenon until the anesthesia is so deep that the circulation stops. With a number of other drugs we have tried to produce changes in the microphone effect; *the substances tested were: salicylic acid, quinine, allypropynal, adrenaline, prostigmine, magnesium sulphate, and calcium.* It was also tested whether large doses of hypertonic and physiological saline solution injected intraperitoneally would affect the potentials.

The *salicylic acid* was administered intraperitoneally as sodium salicylate in a 10 % solution in doses of 5 ml at a time, at intervals of 5—10 minutes, up to 80 ml in all. During this procedure no change whatever was observed in the threshold values at the various frequencies ("the electrical audiogram").

Allypropynal (allylisopropylbarbituric acid) was injected intraperitoneally in a 5 % solution, 10 ml in all, *prostigmine* "Roche" was likewise injected intraperitoneally, 2 mg in all (4 ml), *adrenaline* was administered intraperitoneally (1 ml of a 1 ‰ solution) and, finally, *calcium* "Sandoz" was injected intraperitoneally, 5 ml in all of a 10 % solution; none of these substances influenced the microphone effect.

HUGHSON and CROWE (1933) found that the microphone effect was reduced after intravenous *injection of a hypertonic saline solution* (30 %) which produced a fall in the intralabyrinthine pres-

sure, while distilled water, which increases the intralabyrinthine pressure, did not affect the phenomenon. With large doses of physiological saline solution, up to 80 ml in all, injected intraperitoneally, we found no change in the potentials; after injection of hypertonic saline solutions (NaCl—30 %), up to 40 ml, we observed no reduction of the potentials before the incidence of death; but immediately after the injection the potentials grew very irregular. *Magnesium sulphate* in a 20 % solution yielded a similar result.

Discussion.

It must be admitted that the importance of the microphone effect for hearing has not been conclusively proved; it is possible that the very strong potentials are an adventitious physical phenomenon without any bearing on the transference of sound to the auditory nerve; but this seems extremely unlikely.

It has been maintained that by leading off from much simpler systems than the cochlea, even from a piece of moist cotton wool, a microphone effect can be obtained; we have therefore tried to lead off potentials from a piece of cotton wool saturated with a physiological saline solution. This, however, only proved possible when the cotton wool was placed in the same room as the intensifier; but these potentials were many times weaker than the WEVER-BRAY effect and could only be observed with very strong whistling tones. They did not set in until about 1 second after the sound stimulus and persisted for about 1 second after the cessation of the sound stimulus (bad damping). A closer investigation showed, however, that these potentials were not derived from the cotton wool at all, but were due to the fact that the strong whistling tones directly influenced the intensifier and made it resound. It was understandable, therefore, that in these experiments the intensifier had to be placed in the room where the experiment was carried on.

The exceedingly strong and pure microphone effect which can be elicited from the cochlea renders it extremely probable that this phenomenon is an absolutely necessary link in the mechanism of hearing. Our experiments have shown that the microphone effect on guinea-pigs whose cochlea very much resembles that of man comprises a range of frequencies from about 30 to 14,500 cycles; this corresponds fairly well with the audible range of frequencies for man. It is therefore likely that the guinea-pig can

hear tones, at any rate within this range, and probably only within this range.

We have found the microphone effect extremely resistent to a number of very different drugs; this might be expected, seeing that hearing, as is well known, is only affected by a very few; of those here tested especially salicylic acid and quinine. But as we have previously found (JUUL, 1943), the point of attack of these substances presumably lies centrally to the organ of Corti. Since the microphone effect probably arises in the organ of Corti or peripherally to it (the Reissner membrane?) the results arrived at are understandable. For the hearing of the individual it is of course of the greatest importance that the microphone effect is not affected by drugs.

Our experiments with ascaridol may clear up where the microphone effect originates.

Summary.

1) Our investigations on the microphone effect on guinea-pigs have been performed with a new technique; the anode used being a freshly chloridised silver wire applied directly to the apex of the cochlea. This method seems to yield better results than the methods previously used.

2) The microphone effect on guinea-pigs comprises at any rate a frequency range of from about 30 to 14,500 cycles. The "electrical audiogram" taken on guinea-pigs corresponds fairly closely to the normal audiogram taken on human subjects.

3) The microphone effect is quite unaffected by urethane, salicylic acid, quinine, allypropynal, adrenaline, prostigmine, and calcium. Large quantities of physiological saline solution injected intraperitoneally does not influence the microphone effect. After injection of 30 % NaCl intraperitoneally irregular but not reduced potentials are observed. Reduction only sets in simultaneously with death. Magnium sulphate in a 20 % solution yielded the same result.

4) At the incidence of death the microphone effect is immediately reduced to about 1/5 of its original size and during the succeeding hours it decreases quite gradually till finally it completely ceases.

5) The microphone effect must presumably be regarded as a necessary link in the perception of sound. It is probable that tones

can be heard by the animal within the same range of frequencies as it is possible to demonstrate the microphone effect with the technique here employed.

References.

ADRIAN, E. D., J. Physiol. 1931. *28.* 71.

BRAY, CH. W., and M. LAWRENCE, Ann. Otol. Rhinol. Lar. 1941. *50.* 317.

DAVIS, H., A. J. DERBYSHIRE, M. H. LURIE, and L. J. SAUL, Trans. Americ. otol. Soc. 1933.

DAVIS, H., S. DWORKIN, M. H. LURIE, and J. KATZMAN, Laryngoscope 1937. *47.* 435.

EYSTER, J. A. E., T. H. BAST, and M. R. KRASNO, Ibidem 1937. *47.* 461.

FALBE-HANSEN, J., Clinical and experimental histological studies on effects of salicylate and quinine on the ear. Diss. Copenhagen 1941.

HALLPIKE, C. S., and A. F. RAWDON-SMITH, J. Physiol. 1934. *83.* 243.

HUGHSON, W., E. THOMPSON, and E. G. WITTING, Laryngoscope 1937. *47.* 480.

HUGHSON, W., and S. J. CROWE, Acta otolaryng., Stockh. 1933. *18.* 291.

JUUL, A., Ibidem 1943. *31.* 386.

LURIE, M. H., H. DAVIS, and A. J. DERBYSHIRE, Ann. Otol. Rhinol. Lar. 1934. *43.* 321.

OKA, N., Jap. J. med. Sci. VI. Pharmacol. 1929. *3.* 201.

SAUL, L. J., and H. DAVIS, Arch. Neurol. Psychiat., Chicago 1932. *28.* 1104.

STEVENS, S. S., and H. DAVIS, Hearing, its Psychology and Physiology, New York 1938.

WEVER, E. G., and C. W. BRAY, J. exp. Psychol. 1930. *13.* 373.

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On Serum Copper. II. Technique of Analysis.

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Since O. WARBURG's discovery (1927) of copper as a normal constituent of human serum, much work has been done in order to find a simple method for the microdetermination of copper. Among these methods, the best seems to be the colorimetric method, depending on the colourformation between copper and diethyl-dithiocarbamate.

In 1908 DELÉPINE described the constitution of diethyldithiocarbamate (thiocarbamate) and its ability to form with very weak copper solutions a very intense golden brown to black colour. DELÉPINE also showed that this coloured substance is only slightly soluble in water, while it is easily soluble in ether and benzene. DELÉPINE's work was forgotten, and the reaction of thiocarbamate with copper was rediscovered by CALLAN and HENDERSON (1929), and therefore the reaction at one time was called the CALLAN-HENDERSON reaction (TOMPSETT 1934).

A proper method for determination of copper with thiocarbamate in organic material must include: 1) Liberation of copper as cupric ions, 2) Removal or deionization of iron, 3) Formation of the coloured substance on addition of thiocarbamate, 4) Colorimetry, eventually after solution in a particular solvent.

Liberation of Copper.

In dry ashing the temperature must not exceed 400° C., if loss of copper is to be totally avoided (MCFARLANE 1932), and therefore wet ashing is quicker and safer. For this, sulphuric acid with perchloric acid, and sometimes also nitric acid, are generally used. BRAUN and SCHEFFER[1] (1940) performed the ashing in test-tubes

of 25 ml, but in order to avoid any splattering from the tubes on vigorous boiling, the author has preferred to use microkjeldahl flasks of 40 ml. As nitric acid positively ensures against deficient ashing, 0.5 ml is used together with 0.2 ml sulphuric acid and 0.1 + 0.1 ml perchloric acid for 1 ml serum. For 1 ml of blood 0.25 ml sulphuric acid, 1 ml nitric acid and 0.5 + 0.5 ml perchloric acid will suffice. When the heating is commenced cautiously, destruction proceeds smoothly. Towards the end of the destruction, a more intense heat is employed to remove the perchloric acid and decrease the amount of sulphuric acid.

Deproteinization of serum by means of trichloroacetic acid, first used by LOCKE et al. (1932), who otherwise followed the original method of MCFARLANE (1932), has been claimed by TOMPSETT (1934) and HEILMEYER et al. (1941) to give the same result as ashing both in serum and whole blood. Still, BRAUN and SCHEFFER (1940) and EDEN and GREEN (1941) prefer wet ashing.

In a preliminary experiment with determination of the copper contents of 19 sera, duplicate determinations were made on each serum after ashing and after treatment with trichloroacetic acid. On comparison of the averages of the two duplicate determinations, the difference in no instance exceeded 2 %. Similar agreement was found in a series of determinations on 19 samples of oxalated blood. The deproteinization was performed on diluted serum (blood), 1 ml of serum (blood) being mixed thoroughly with 3 ml of water prior to the addition of 1 ml trichloroacetic acid (20 %). After shaking, the mixture was left standing for 10 min., then centrifuged (3000 revolutions) for 10 min., and the copper content then determined as described below on 3 ml of the clear supernatant fluid. The agreement between corresponding values, obtained through ashing or trichloroacetic acid treatment, shows that it is not necessary to add hydrochloric acid prior to the deproteinization, as it is in determination of the iron in contrast to the statement made by SCHMIDT (1939).

Inactivation of Iron.

As iron also forms a coloured compound with thiocarbamate, it has to be removed or deionized before the addition of thiocarbamate. Better than the precipitation of the iron as ferric hydroxide (CALLAN and HENDERSON 1929) and filtration or precipitation as pyrophosphate (DRABKIN and WAGGONER 1930) is the formation of a soluble complex between iron and pyrophosphate (MCFARLANE) or citrate (HADDOCK and EVERS 1932) at alkaline reaction.

While some authors (McFARLANE, TOMPSETT) employ a small excess of ammonia and heat for 10—15 minutes on a water bath, others (EDEN and GREEN 1940, HEILMEYER et al. 1941) prefer a great excess of ammonia without heating, or sodium hydroxide (LESNE et al. 1936), or potassium carbonate (BRAUN and SCHEFFER 1940). Strange to say, none of the authors emphasize that ammonia is not particularly suitable for this purpose, if amyl alcohol is used for removal of the coloured compound. Ammonia dissolves readily in amyl alcohol and with the carbon dioxide of the air it may form a cloudy precipitate of ammonium carbonate in the alcohol, making photometric reading most uncertain. Therefore hydroxides or carbonates of the alkaline metals — lithium excepted — are preferable. It proved quite satisfactory to add potassium carbonate and sodium pyrophosphate dissolved in the same solution (BRAUN and SCHEFFER). In the presence of greater amounts of iron, the fluid on addition of water and pyrophosphate-carbonate solution first becomes opaque, but in 5—10 min. it turns clear. Pyrophosphate solution with ammonia instead of carbonate shows similar features. The thiocarbamate is not added till the fluid is clear.

Addition of Thiocarbamate. In 1 ml of deproteinizated blood or serum or 1 ml of ashed serum the amount of iron will be less than 5 γ. By examination of blank samples with and without addition of 5 γ of iron it can be demonstrated that 0.5 % pyrophosphate in the reaction mixture is sufficient to prevent any colour formation between the iron and the thiocarbamate in a concentration of about 0.02 % (0.4 ml of 0.5 % thiocarbamate solution to 8 ml). In acid ashing of 1 ml of whole blood, however, the amount of iron is about 0.5 mg. When thiocarbamate here is added without careful shaking of the solution, the formation of a colour between iron and thiocarbamate will interfere (EDEN and GREEN 1940). Still better than adding a weak solution of thiocarbamate under constant shaking is the use of thiocarbamate dissolved in a 4 % pyrophosphate solution. This solution keeps just as well as does the ordinary aqueous solution for a month. When the thiocarbamate-pyrophosphate solution is added during shaking, as much iron as 1 mg does not disturb.

The smallest amount of thiocarbamate giving maximum colour with 1 γ copper is about 0.05 mg. The author has used 0.4 ml of a 0.5 % solution, i. e. 2 mg. Various authors use from 1 to 10 mg.

Solvent. In 1932 McFARLANE first used amyl alcohol for separation of the coloured compound. McFARLANE states that the amyl

alcohol has to be very pure, as otherwise the colour will be unstable. From this work amyl alcohol appears to be the solvent commonly used for the copper colour. The mutual solubility between water and amyl alcohol makes it necessary to keep the proportion between the alcohol phase and the aqueous phase constant. Experiments have shown that when 8.5 ml of water are shaken with 2 ml of amyl alcohol, the volume of the alcohol phase will be altered less than 1 %. Further, repeated shaking of a fraction of the water phase with amyl alcohol has shown that, at the most, 0.3 % of the whole colour remains in the water phase. Other solvents, e. g. dilute ethyl alcohol (SCHMIDT 1939) or carbon tetrachloride (HADDOCK and EVERS 1932, NYDAHL 1939) offer no particular advantage. Carbon tetrachloride yields no stronger colour than amyl alcohol.

Separation of Amyl Alcohol. In order to remove the tiny droplets of water which remain suspended for some time in the amyl alcohol, centrifuging is safer than the use of "copper-free" filter-paper. Centrifuging for 30 min. (3,000 revolutions) in ordinary centrifuging tubes gives no measurable concentration of the amyl alcohol, which has been demonstrated by photometry of the colour of the solution before and after centrifuging.

Colorimetry. After centrifuging, the amyl alcohol is transferred by pipette directly to the 50 mm long microcuvettes of the Pulfrich photometer; the ordinary photometer lamp and filter 43 are employed; amyl alcohol is used for comparison.

Calculatory factor. Through 28 analyses of dilutions of a stock solution — 0.3926 g $CuSO_4$, 5 H_2O p. anal. in 1 liter glass-distilled water-ranging from 0.0005 to 0.002 mg Cu, the calculatory factor, i. e., the factor which multiplied with the extinction gives the copper content in γ%, has been found to be 0.1993 ($s = \pm 0.00172$). For 0.001 mg Cu in the analysis (2 ml amyl alcohol), or 0.100 mg%, the average extinction $E_{1.50\ mm} = 0.5017$ (*0.502*).

Control Experiments. In two ways I have shown that the colour estimated by photometry is due to the copper content of the serum and to nothing else. In the first place, an absorption curve from a serum analysis, obtained by plotting the extinction with all the filters of the photometer, has exactly the same form as the corresponding curve obtained from a pure copper solution. In the next place, the development of the colour may be prevented by addition of KCN (0.4 ml of a 1 % solution), as the cyan ion removes the cupric ion by forming cuprocyanide.

That the analysis is quantitative is evident from Table 1.

Table 1.

Recovery of Cu added to Sera with Known Cu Content.

	E			E	Recovery
1 ml Serum	.519, .522, .526, .520 } .52175	1.0 Cu added		1.027, 1.022, 1.032, 1.027 } 1.027	$\frac{\text{Found } 100}{\text{Expected}}$ = 100.33 %
1 » »	.699, .697 } .698	0.5 » »		.955, .951 } .953	» = 100.42 %
1 » »	.595, .592 } .5935	0.5 » »		.845, .842 } .8435	» = 99.88 %
1 » »	.600, .609 } .6045	0.5 » »		.860, .857 } .8585	» = 100.35 %
1 » »	.477, .480 } .4785	0.5 » »		.731, .728 } .7295	» = 100.00 %

Blank Value. As even the best analytical preparations contain a trace of copper, a blank value is unavoidable. As a rule, the blank value corresponded to 0.15—0.20 γ Cu or about 15 % of the normal copper content of 1 ml of serum. Of the reagents used, ordinary conc. sulphuric acid was found to contain less copper than analytical sulphuric acid. Isoamyl alcohol that has been used once may be used again after fractionated redistillation 3 times. Potassium carbonate and sodium pyrophosphate are purified separatedly in concentrated solution by addition of thiocarbamate and shaking with amyl alcohol. After evaporation and heating in an electric oven to 550° for 24 hours the thiocarbamate is clarred; the carbon is removed by filtration through suction filter G4, after the reagents are dissolved for use. Only ordinary distilled water was employed, but redistilled water may be required.

Every working day both blank value and a standard solution were determined.

Reagents:

Sulphuric acid, concentrated.
Nitric acid pro anal.
Perchloric acid pro anal. (sp. gr. 1.65).
Trichloroacetic acid, redistilled pro anal.
Isoamyl alcohol pro anal., boiling point 130°.

Pyrophosphate-carbonate solution: 250 g. potassium carbonate dissolved in about 450 ml distilled water. After cooling, 20 g sodium pyrophosphate (anhydrous) is dissolved in this solution, and the flask is filled to the 500 ml mark (then filtration if required).

Sodium diethyl-dithiocarbamate pro anal., 0.5 % solution in 4 % sodium pyrophosphate.

Contamination. In order to avoid errors, the glass utensils have to be cleaned with chromic acid. As rubber contains a great deal of copper, water which has been standing in a rubber tube must not be employed, nor rubber stoppers in the tubes containing the blood specimens. Paraffined corks proved suitable. The blood was taken from the veins by means of stainless steel needles *without stilette*. After withdrawal of the blood, the needle was cleaned with distilled water and dried at 100° (in an ordinary needle box).

Determination after Ashing. In a microkjeldahl flask, 1 ml. of serum together with 0.2 ml sulphuric acid, 0.5 ml nitric acid and 0.1 ml perchloric acid is heated — at first gently, then after 10 min. and addition of a second dose of 0.1 ml perchloric acid intensely — till only a few drops of clear fluid are left. After cooling, 6 ml of distilled water are added, then 2 ml of pyrophosphate-carbonate solution and — after shaking, when the fluid is perfectly clear — 0.4 ml (8 drops) thiocarbamate and 2 ml of amyl alcohol. After very thorough shaking in the flask, the mixture is poured into an ordinary centrifuging tube and centrifuged for 10 min. Then the supernatant fluid is transferred directly (by means of a pipette) to be microcouvette for photometry. Ashing of 1 ml of blood is carried out with 0.25 ml sulphuric acid, 1 ml nitric acid and 0.5 + 0.5 ml perchloric acid.

Determination after Trichloroacetic Acid Precipitation. One ml of serum (blood) is mixed thoroughly with 3 ml distilled water; then 1 ml of 20 % trichloroacetic acid is added. After additional shaking, the mixture is left standing for 10 min., and then centrifuged for 10 min. 3 ml of the supernatant fluid is transferred to a microkjeldahl flask; 1 ml of pyrophosphate carbonate is added, then 4 ml. of distilled water; then the further procedure is the same as after ashing. After subtraction of the blank value, the result has to be multiplied by 1.67 ($\frac{5}{3}$).

Acid ashing has proved superior because of its simplicity and the easy cleaning of the utensils.

Summary.

Description is given of the author's modification of McFARLANE's method for determination of the copper content of blood and serum by means of diethyl-dithiocarbamate.

Experiments show that acid ashing and trichloroacetic acid treatment give the same results.

For alkalinization the addition of ammonia in excess is unsuitable as it may be precipitated in the amyl alcohol as ammonium carbonate. In determinations on whole blood the presence of iron after ashing may cause an error which is avoided most easily by adding the thiocarbamate dissolved in a solution of pyrophosphate.

References.

Braun, L., and L. Scheffer, Biochem. Z. 1940. *304*. 397.

Brochner-Mortensen, K., and C. Olsen, C. R. Lab. Carlsberg, Sér. chim. 1940. *23*. 235.

Callan, Th., and J. A. R. Henderson, Analyst 1929. *54*. 650.

Delépine, M., Bull. Soc. Chim. 1908. *3*. 652.

Drabkin, D. L., and C. S. Waggoner, J. biol. Chem. 1930. *89*. 51.

Eden, A., and H. H. Green, Biochem. J. 1940. *34*. 1202.

Haddock, L. A., and N. Evers, Analyst 1932. *57*. 495.

Heilmeyer, L., W. Keiderling, and G. Stuwe, Kupfer u. Eisen als körpereigene Wirkstoffe, Jena 1941.

Lesné, E., P. Zizine and S.-B. Briskas, C. R. Soc. Biol., Paris 1936. *121*. 1582.

Locke, A. E. ,R. Main and D. O. Rosbash, J. clin. Invest. 1932. *11*. 527.

McFarlane, W. D., Biochem. J. 1932. *26*. 1022.

Nydahl, F., Z. anal. Chemie 1939. *116*. 315.

Schmidt, H. G., Biochem. Z. 1939. *302*. 256.

Tompsett, S. L., Biochem. J. 1934. *28*. 1544.

Warburg, O., and H. A. Krebs, Biochem. Z. 1927. *187*. 255.

Addendum: With this method the serum copper values of 100 normal, non-fasting women and 30 men have been found to lie within the range from 75 to 165 γ%.

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The Selection of Food.

II. Need and Desire for Proteins.

By

ERIK M. P. WIDMARK.

Received 18 February 1944.

The question as to whether lack of nitrogen in food produces a desire for foods containing proteins demands a critical consideration of the selection tests to which experimental animals are subjected.

As already stated in Communication I (1944) we must consider whether the substance in question in itself exercises a positive or negative chemo-taxis even before there is a lack of it in the food.

With protein experiments in *fully-nourished animals* three alternatives can be imagined:

a) the protein tested in the selection experiment possesses a negative chemo-taxis,

b) the protein, in its taste effects etc., is neutral. The quota will lie round about 50,

c) the protein has a positive chemo-taxis effect. Even the animals fully supplied with nitrogen prefer the food containing this protein. The quota will exceed 50.

If the lack of nitrogen increases the desire for protein it may be expected that in every case the quota will be higher than those obtained from fully-nourished animals. In alternative a) the quota values need not rise above 50, because there can be a certain aversion from the food substance.

The following experiments, carried out according to the method described in Communication I, seem to show that lack of nitrogen produces an increased desire for protein in the experimental

animals: rats. Naturally the investigation is in no way exhaustive, but rather preparatory to later, more exact, investigations. Under present conditions there have been difficulties in testing the effects of a large number of pure proteins; nor have the experiments been placed in relation to the metabolism of nitrogen in the animals. Such estimates will certainly in the future be specially illuminating in regard to the starting-point of the desire for protein. Investigations having as their object the need for the vital amino-acids (called by the author "vitamino-acids") have not yet been carried out, and therefore we do not yet know whether the animal's organism has the capacity to discover them when mixed with different food substances. In this there lies ready to hand a large and interesting sphere for nutritional investigation, and I hope to return to it.

Tests with Casein.

9 animals, 6 males and 3 females, taken direct from the mother (circa 1 month old, average weight 37 g).

Foods: a, rich in protein:

wheat starch	62 g per 100 g dry food
casein (not free of water)	20
yeast	6
mixed salts	6
arachis oil	6
Cod liver oil	0.6

N contant ca 2.74 % = 17.1 % protein.

b, poor in protein:

the same as *a*, only with the difference that the casein was replaced by wheat starch: then the starch content is 82 % of the dry food.

N content 0.53 % = 3.3 % protein.

70 g water was mixed into 100 g of the dry food.

During the first 5 days the animals were allowed to choose between the two foods. As is shown, these animals taken direct from the mother and therefore presumably having received an optimum supply of protein, show a marked negative chemo-taxis for the food rich in casein (fig. 1). All the quotas lie below 50, between 23 and 34 (= 5 days).

From the 6th to the 14th day the animals received food poor in nitrogen (*b.*, N = 0.53 %). After that, from the 15th to the 26th day, they were again allowed to choose between the two foods (but with 2 short periods of 2 and 1 day with food poor in protein). The negative chemo-taxis for casein had now disappeared. The lack of nitrogen during the 9 days had produced an increased desire for protein. All the quotas now lie above 50. The highest value is 83.

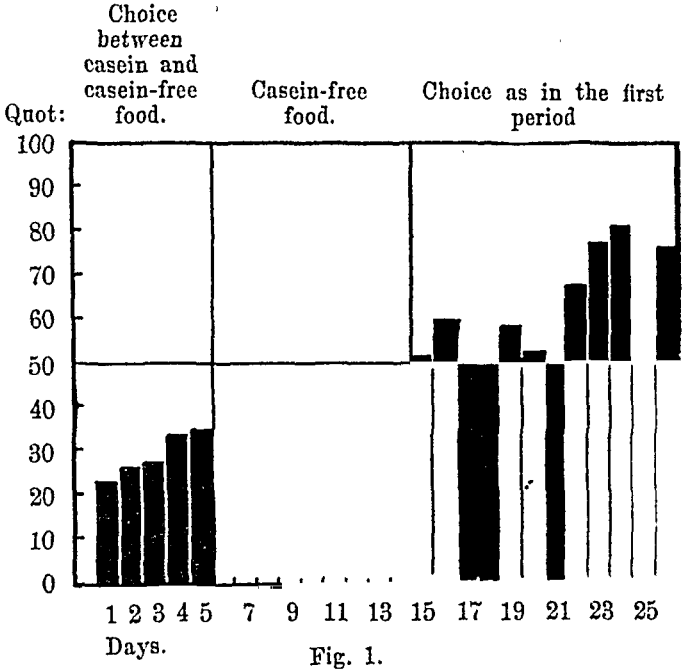

Fig. 1.

The figure here given represents the average values of the quotas in all the animals.

It is interesting to note that there are individual variations in the animals. An examination of each animal shows that *they all react to lack of protein in the same way in principle:* it induces an increased consumption of the nitrogenous food. Yet there were individual differences, which is not at all surprising. The study of the consumption habits of the different individuals gives an opportunity to distinguish the different types, which is perhaps simpler and more objective than any other method. We shall return to this question later.

As examples of the individual differences the quotas are given from the two extreme cases in this experiment, namely for animal 71, (female) and animal 69, (male).

Table showing 2 extreme cases as regards chemo-taxis for casein before and after a period of N-starvation.

Test days	Individual quota Animal 71 fem.	Animal 69 male
1	16	38
2	15	49
3	2	31
4	2	54
5	6	52
6—14	N Starvation	N Starvation
15	58	55
16	94	66
17	—	—
18	—	—
19	87	67
20	95	32
21	—	—
22	63	71
23	79	82
24	83	81
25	93	78
26	81	62

No 71, a female, shows a specially marked negative chemotaxis for casein food (average quota 8). After 48 hours it practically refuses to select this food. After N-starvation, however, the aversion has completely disappeared. The consumption rises to an average quota of 81.

No 69, male, shows a very inconsiderable aversion for casein. The average quota before the N-starvation is 45. After it the quota rises to an average of 66.

The varying intensity in the reaction after nitrogen starvation clearly invites the conclusion that the intense desire for nitrogen in number 71 is produced by the low nitrogen consumption during the first 5 days, while number 69 in this preliminary period maintained a rather considerable nitrogen consumption, resulting in less desire for protein. The explanation is plausible and consistent but demands more experimental material for its confirmation.

The change in the animals' selection of food must be connected with changes in the bodily condition on account of the lack of nitrogen. The food in each of the two cups is the same and tastes the same in the introductory experiments when the animals are fully nourished as in those made with the same animals after a period of nitrogen starvation.

In principle the same result has been obtained in a second experiment with casein which is represented in fig. 2.

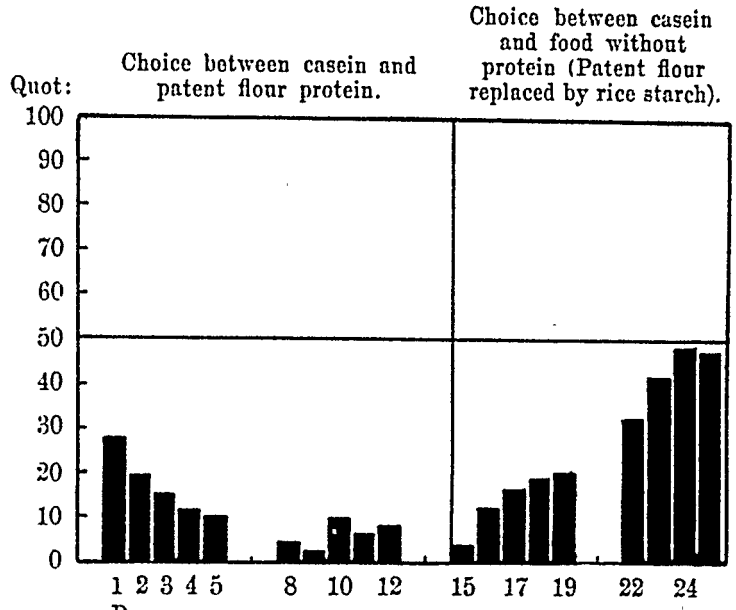

Fig. 2.

This experiment was carried out as follows:

7 rats, reared on mixed food, rich in protein (skim-milk etc.) were allowed to choose between the following 2 foods:

a) complete food with 21.2 % casein and patent flour as a carbohydrate source. N = 5.2 %.

b) the same food but without casein. The casein was replaced by a corresponding amount of patent flour. N = 2.1 %.

A certain amount of N was thus already supplied by the flour; the *a* food (casein food) was, however, more than double as rich in nitrogen.

During the first 12 days the animals showed a specially marked aversion from the casein; the quota was even lower on the average than in the previous experiment.

After that the flour was replaced by rice starch *in both foods*. The N content fell to 3.9 and 0.6 % respectively. Thus, the casein-free food became almost wholly lacking in protein.

It is now seen that from the 15th to the 25th day the quota shows a continuous rise. The casein becomes more and more desirable, so that the quota approaches 50. Yet a certain inhibition continues to assert itself. The consumption during the 22nd to the 25th days ought, however, to cover the animals' need for protein: 1.6, 2.0, 2.1 and 2.0 g N respectively.

Experiments with Serum-Albumin.

Circumstances prevented more than a small quantity of serum-albumin being at our disposal (Merck). The experiments were therefore only carried out on a limited scale.

4 rats (2 males and 2 females), taken direct from the mother at 1 month (average weight when the experiment began 54 g), were given the food described in the previous experiment, only that serum albumin was substituted for the casein. N content of the albumin food was 2.7 %, of the albumin-free food 0.53 %.

Only 3 preparatory experiments could be made. After that the animals were put on the N-poor food. The results are shown in the following table:

Table showing the selection of food containing serum albumin after 10 days N-starvation.

Experiment day	Quota
1	41
2	50
3	50
4—13	N-starvation
14	55
15	61
16	59

This experiment shows in principle the same result as the casein experiments. The material, however, is not sufficiently large for valid conclusions. For the 3 day periods before the N starvation the 2 males showed on the average quotes 38 and 33, after it 61 and 57. The females had, before the N starvation, secured for themselves a relatively rich supply of N by the quotas 52 and 67. After it they were 50 and 63 respectively.

The experiments differ from the casein experiments chiefly in that the negative taxis in the fully-nourished animals is less marked in the males and is absent in the females.

Experiments with Wheat Gluten.

In these experiments the same food was used as in that with albumin, only with the difference that wheat gluten was substituted for the albumin. 9 rats (5 males and 4 females), one month old, taken direct from their mothers, were allowed to choose between the food containing gluten and that without it.

In 6 days the following average figures for quotas were obtained:

Experiment day	Quota
1	51
2	70
3	62
4	68
5	60
6	71

These albuminous substances thus showed a positive chemotaxis already without a Nitrogen starvation period. The individual variations are not specially noticeable. Calculating the average quota for each animal, one is found to have the quota 50. The quotas of the others varied between 56 and 77.

As the wheat gluten is attractive to the animals already before there is any lack of nitrogen, these proteins are not particularly suitable for testing whether such a lack increases the desire for protein. Further experiments in this matter have not been made.

Summary.

1) In fully-nourished animals casein shows a negative chemotaxis. After some days without nitrogen in the food this aversion decreases considerably. The quotas approach 50 and often rise considerably above it, so that the animals in certain cases practically speaking consume only the casein-rich food, while they leave the nitrogen-poor food almost untouched.

2) In fully-nourished animals serum albumin is almost neutral (quota value round about 50). After N-starvation the average values for the quotas rise.

3) In fully-nourished animals the wheat gluten has already a marked positive chemo-taxis (quota value up to 71).

4) The changes in the animals' selection of food must be connected with changes in the bodily condition on account of lack of nitrogen. The food in the 2 cups is the same and has the same taste in the preparatory experiments when the animals are fully nourished, as in those carried out with the same animals after a period of nitrogen starvation.

Reference.

WIDMARK, E. M. P.: Acta physiol. 1944. 7.

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Isolation from Urine of a Volatile Base with Nicotine-like Action.

By

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Received 22 March 1944.

In 1908 ABELOUS and BARDIER found a pressor base in urine which they called urohypertensin. The active principle was later thought to be identical with *iso*-amylamine, isolated by BARGER and WALPOLE (1909) from putrid meat. BAIN (1914) isolated a pressor base from urine and concluded from the chemical data and the biological action that it was *iso*-amylamine. In urine from normal subjects EULER and SJÖSTRAND (1942) found greater amounts of a pressor principle — which was believed to be identical with BAIN's substance — than in urine from hypertensives.

Recently HELMER, KOHLSTAEDT and PAGE (1939) have drawn attention to the fact that nicotine may appear in smoker's urine and give rise to a pressor action in urine extracts.

In a study of the pressor activity of the urine of various animals it was found that ether extracts of cow's urine exerted a strong pressor action on the blood pressure of the cat. As certain facts indicated that the active principle was neither *iso*-amylamine nor nicotine an attempt was made to isolate and characterize the pressor substance.

Preparation of the Substance.

Cow's urine was concentrated in vacuo at a slightly acid reaction and subjected to continuous ether extraction at pH 10.

The active substance was removed from the ether with acidified water and steam distilled at pH 10. The distillate was neutralised with picric acid and the active substance purified by fractionated crystallization.

The active principle was isolated as a cristalline picrate (Fig. 1) which melted at 141—142°. Assuming that the base combines with 1 equivalent of acid, its molecular weight would be approximately 80. The free base is volatile and passes in the first portions when subjected to steam distillation. Prolonged heating in alcaline solution exceeding pH 10 inactivates the substance.

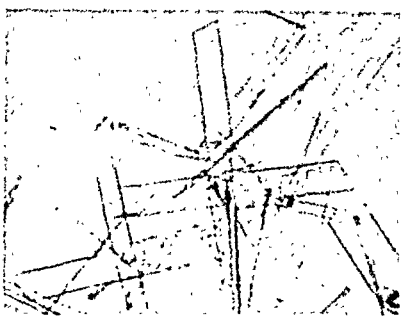

Fig. 1. Microphotograph of picrate crystals of the active base from ethyl-alcohol —water. (× 20).

Biological Actions.

In the biological assay during the purification work, it proved useful to employ nicotine as a standard, since the actions of the two substances were very similar on the blood pressure of the cat and on the isolated intestine of the rabbit (fig. 2). Certain differences in action were noted, however, as regards the details in the pressor or motility curves. 1 mg of the crystallized picrate corresponded to the action of about 15 μg nicotine base. 1 mg of the free base approximately equalized the action of 0.05 mg nicotine, though variations in the relative action were observed in some animals.

The pressor action was either unchanged or increased by cocaine in distinction to *iso*-amylamine (TAINTER, 1933). The pressor action was reduced, but not abolished, by ergotamine in doses of 1—2 mg/kg in the cat.

Occasionally the action on the blood pressure of the cat or on the isolated rabbit's intestine was very weak, but in these cases

nicotine too, had a correspondingly slight action, indicating that both substances have a similar mechanism of action.

Even on the respiration the action of the new base closely resembled that of nicotine, in that respiration was strongly reinforced reflexly. This action disappeared after denervation of the chemoreceptor regions.

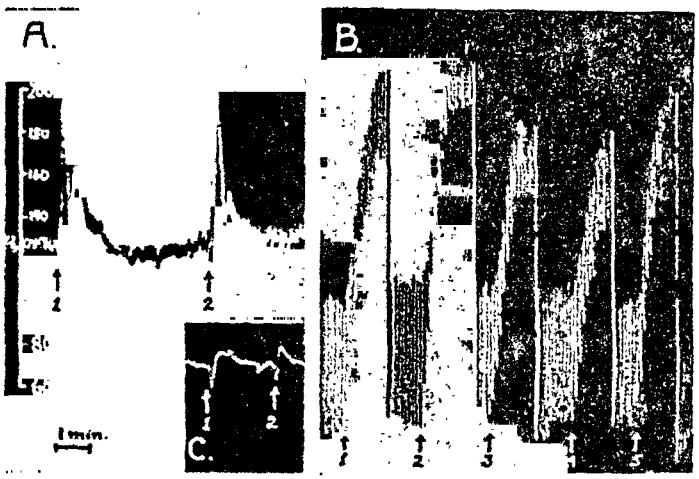

Fig. 2. A. Blood pressure, cat, chloralose. 1) 3 mg crystalline picrate, 2) 0.05 mg nicotine. B. Rabbit's isolated intestine. 1-3-5) 0,01 mg nicotine, 2 and 4) 1 and 0.5 mg crystalline picrate respectively. C. Rabbit's blood pressure, urethane. 1) 0.1 mg nicotine tartrate, 2) 3 mg crystalline picrate.

It is inferred from these observations that the active principle has a general synaptotropic action like that of nicotine. The presence of a substance of this kind in normal urine may be of physiological significance. It is also evident that it differs from the other physiologically occurring synaptotropic substance, acetylcholine, as there is no indication of a muscarine-like action.

Summary.

A physiologically active volatile base has been isolated as picrate from cow's urine.

Qualitatively its action resembles that of nicotine on the blood pressure of the cat and rabbit, the motility of the isolated intestine of the rabbit and the respiration on the cat, though it is about 20 times weaker.

The action is not abolished by cocain and is partly inhibited by ergotamine.

The possible physiological significance of the occurrence in normal urine of a substance with a general synaptotropic action is pointed out.

I wish to acknowledge the valuable technical assistance of Mr E. ÖSTLUND in connection with this work.

References.

ABELOUS, J. E. and E. BARDIER, J. Physiol. Path. gén., 1908, *10*, 627.
BAIN, W., Quart. J. exp. Physiol., 1914, *8*, 229.
BARGER, G. and G. S. WALPOLE, J. Physiol., 1909, *38*, 343.
EULER, U. S. v. and T. SJÖSTRAND, Nature, 1943, *151*, 168.
HELMER, O. M., K. G. KOHLSTAEDT and I. H. PAGE, Heart, 1939, *17*, 15.
TAINTER, M. L., Arch int. Pharmacodyn., 1933, *46*, 192.

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Further Studies on the Gastric Secretory Excitant from the Pyloric Mucosa.

By

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Received 12 January 1944.

Experimental facts are in full agreement with the conception that the gastric phase of gastric secretion is mediated by a humoral mechanism. Experiments concerning the origin, chemical nature and the mode of liberation of the active principle or principles have however given conflicting results.

Many facts suggest that the humoral principle is liberated by some process confined to the pyloric mucosa. In experiments on dogs it was shown by several members of the Pavlov school that chemical or mechanical stimulation of the mucosa of an isolated pyloric pouch initiated gastric secretion in the main stomach (GROSS 1906, SAVITSCH and ZELIONY 1913 and others). These results were confirmed by LIM, IVY and MACCARTHY 1925, who observed a slight gastric secretion also after mechanical stimulation of the mucosa of the main stomach. From these and later experiments (GREGORY and IVY 1941) IVY and his collaborators conclude that the hormonal mechanism is mediated also by other mucosal regions than that of the pylorus. Experimental evidence presented by UVNÄS (1942) indicates that the cephalic phase of gastric secretion is mediated by a neuro-hormonal mechanism, the hormonal agent being liberated from the pyloric mucosa by vagal impulses.

Since the work of EDKINS (1906) many attempts have been made to isolate a gastric secretory excitant from the pyloric mucosa. Active mucosal extracts were obtained by POPIELSKI

(1909), EMSMANN (1912), EHRMANN (1912), KEETON and KOCH (1915), LUCKHARDT et *al.* (1920), and others. As active extracts were obtained from various regions of the alimentary canal as well as from other tissues and organs, the hormonal nature of the active pyloric principle was doubted. In 1932 SACHS, IVY, VANDOLAH and BURGESS were able to isolate crystalline histamine from extracts of the pig's pyloric mucosa. According to these authors their experiments offered strong evidence in favour of the view that in *acid* extracts of the pyloric mucosa histamine is the sole secretory excitant which is active when the extracts are introduced subcutaneously. According to GAVIN, MCHENRY and WILSON (1933) the fundic mucosa also contains histamine. 80 per cent of the total histamine content of the stomach mucosa was found in this region.

In a preliminary report KOMAROV (1938) claimed to have extracted from the pyloric mucosa of dogs a gastric secretory excitant not identical with histamine. The active agent was obtained in the protein fraction of the extracts. In IVY's laboratory these findings could not be confirmed (IVY 1941). Using Komarov's extraction-technique UVNÄS (1942, 1943), however, found a histamine-free secretory agent predominantly localized in the pyloric mucosa of dogs, cats and pigs. The chemical properties strongly indicated that the active agent was of protein nature.

During the last year we have continued our research into the chemical nature and the physiological properties of the pyloric principle. A later paper by KOMAROV (1942) dealing with this subject did not, unfortunately, reach us until late this autumn, owing to interrupted postal communications.

Experimental.

Method of assay.

Tests of the preparations were made on cats previously starved for 24 hours. The anaesthetic used was a mixture of chloralose and urethane. During a short ether anaesthesia 0.05 gm chloralose and 0.5 gm urethane per kg body weight were slowly injected intravenously. In most experiments the vagi were exposed in the neck and cut. The trachea was cannulated and the oesophagus tied in the neck. The stomach was drained by a thin perforated rubber tube introduced through a glass cannula inserted in the stomach wall just proximal to

the pyloric region. The cannula with the rubber tube was pushed out through a stab wound in the left abdomen. The duodenum was ligated just proximal to Vater's ampulla. After the abdominal wall was closed the animal was placed on its left side, the front half of the body somewhat higher to facilitate the outflow of the gastric juice. Then the animal was left for about one hour before testing was started.

The extracts to be tested were generally suspended in 0.9 per cent NaCl-solution and made slightly acid to Congo by adding a few drops of N HCl. The solution was injected — if necessary after centrifuging — into the iliac vein at a rate of 0.4 ml per minute. After 30 minutes the injection was stopped and the secretion allowed to decline to the basic level. This was usually the case within 30 minutes after the end of the injection.

The secretory volume was measured every 15 minutes. The secretion during the 60 minutes following the starting of the injection was taken as a measure of the activity of the extracts. In order to facilitate the comparison of the activity of the different extracts this was expressed in secretory units. The quantity of active material causing in 60 minutes the secretion of 1 ml strongly acid gastric juice in a cat weighing 2—3 kg was taken as containing a secretory unit. If possible, doses initiating a secretion of 5—15 ml in 60 minutes were chosen. A secretory response below 2—3 ml was taken to be inconclusive.

The total acidity of the gastric juice was determined colorimetrically, phenolphtalein being used as indicator and N/10 NaOH as base.

As observed by Uvnäs (1943) it was seen now and then that the secretory response to the extracts declined during the course of an experiment. The extracts therefore were tested repeatedly and on different animals.

Mode of preparation of the active material.

Our earlier investigations of the distribution and chemical properties of the pyloric principle were carried out on crude preparations simply precipitated from HCl-mucosal extracts by trichloracetic acid. Further studies on the chemical nature and the physiological properties of the active agent necessitated the preparation of a purer material. Owing to the great cost and the difficulty of obtaining sufficient amounts of trichloracetic acid, other ways of getting reliable crude preparations were tried. Of the numerous possibilities investigated, precipitation of the HCl-extracts with 20 per cent NaCl was found to be the most promising.

A. *Crude preparations from pyloric mucosa of cats.*

The pyloric mucosa was removed, minced with scissors, suspended in 10 volumes of N/10 HCl and treated for 30 minutes in a water bath at 100° C. The acid extracts stored in the refrigerator retained their activity unchanged for several days. After centrifuging and filtering through cotton wool the acid filtrate was neutralized with N NaOH

to pH 3—4, and precipitated with an equal volume of 20 per cent trichloracetic acid. 30 minutes later the precipitate was centrifuged off and washed three times with 40—50 volumes of 10 per cent trichloracetic acid in saline, several times with 50 volumes of acetone, once with benzene and 2—3 times with ether, and dried at 37° C. The resulting white powder was then stored in the refrigerator. This mode of preparation always resulted in crude extracts of high activity.

B. *Crude preparations from pyloric mucosa of pigs.*

Extraction with HCl. Stomachs of recently killed pigs were kept on ice and carried from the slaughter house to the laboratory within 1—2 hours after the death of the animals. The stomachs were washed under running tap water and the pyloric mucosa separated. In most experiments the mucosa was ground in a mincing-machine, in others it was only roughly cut into pieces. The mucosa was then thrown into boiling N/10 HCl, 200 ml HCl being used per stomach. The temperature was maintained at about 90° C for about 30 minutes and was then allowed to fall. After a day or two — the mixture could be left at room temperature for days without appreciable loss of activity — the mucosal fragments were removed by filtering through gauze. By adding N NaOH the mixture was neutralized to a pH of 3—4. A precipitate containing inert material was removed by centrifuging for 10 minutes. Care must be taken not to neutralize beyond pH 4, to prevent active material being thrown out with the inactive. The centrifugate was filtered through cotton wool to remove fatty substances floating on the fluid.

Precipitation with NaCl. Sodium chloride was dissolved in the filtrate to give a 10 per cent solution. By adding the same volume of saturated sodium chloride a concentration of about 20 per cent was obtained. A precipitate formed. After about one hour the material was centrifuged just sufficiently to throw the precipitate into a compact layer. 20—30 minutes were necessary. The supernatant fluid was removed and the sediment gathered in a glass vessel. To remove as much water as possible the precipitate was centrifuged once more for 45 minutes. It was then washed several times with 10—20 volumes of acetone. During this procedure the precipitate acquired a tenacious consistency and stuck to the vessel walls. The washing had to be repeated until this consistency had quite disappeared and the precipitate was dry. It was then washed twice with benzene and three times with ether. The residue was dried by air and if correctly prepared a white-yellow, fine powder was obtained. The yield amounted to 4—7 gm dry substance per stomach.

Numerous methods for the purification of the NaCl-precipitate have been tried. Two reliable, simple procedures will be described by which more then 95 % of inert material was removed.

I. *The tannic acid method.*

a) *Precipitation with tannic acid.* The dried NaCl-precipitate was suspended in N/10 HCl for 30 minutes at a temperature of about 90° C

and stirred frequently. 100 ml HCl was used to dissolve a quantity of dry substance corresponding to one stomach. After cooling the undissolved residue was centrifuged off. The fluid was neutralized with N NaOH to a pH of about 3 as described previously and 5 ml of a 5 per cent tannic acid solution added. A grey-white precipitate appeared. This was centrifuged off and washed once with saturated sodium chloride solution, twice with acetone, once with benzene and twice with ether. The precipitate was left to dry by air.

b) *Washing with alcohol.* The active principle was found to be practically insoluble in 80 per cent alcohol. By washing the tannic acid precipitate with acid alcohol a considerable amount of inert material could therefore be removed. An amount of dry substance corresponding to one stomach was suspended in 75 ml 80 per cent alcohol containing 1 ml of 10 N HCl per 100 ml alcohol. The mixture was stirred at room temperature for 15 minutes and the undissolved residue centrifuged off.

c) *Precipitation at pH 8.0.* The wet residue from the above was dissolved in 0.9 % NaCl-solution, 50—75 ml being used for an amount of dry substance corresponding to one stomach. After 15 minutes' stirring at about 40° C any possible undissolved material was centrifuged off. Drops of N NaOH were added to a pH of 8.0. This gave a precipitate containing inactive material which was centrifuged off.

d) *Precipitation with trichloracetic acid.* The clear fluid from the above acidified to pH 3—4 with N HCl, and trichloracetic acid, in substance or in 20 % solution, was added to a final concentration of 10 %. The active agent precipitated was centrifuged off and washed twice in acetone, twice in ether and dried by air.

In some experiments the wet residue from the alcohol-washing was suspended directly in a Sörensen phosphate buffer with a pH of 8.0 and at a temperature of 40° C, the same amount of NaCl-solution being used as in the procedure described above. The undissolved residue was removed by centrifuging and the clear fluid treated as in I d. Both procedures gave highly active preparations.

II. *The trichloracetic acid method.*

a) *Precipitation with trichloracetic acid.* The dried NaCl-precipitate was extracted in N/10 HCl as described under I a. After removing undissolved material and neutralizing to pH 3—4, an amount of 20 % trichloracetic acid solution was added to give a concentration of 10 %. The resulting precipitate was centrifuged down for about 30 minutes and then washed several times with acetone, twice with ether and dried by air.

b) *Washing with alcohol.* Washing the dried precipitate from the above with 80 % acid alcohol was done as described under I b. The procedure in some experiments removed considerable amounts of inert substances. In others it was less effective. In some of the purifying experiments this step was therefore excluded.

c) *Precipitation at pH 8.0.* The procedure was identical with that

described under I c. The dried trichloracetic acid precipitate from II a or the wet residue from II b was dissolved in NaCl-solution or phosphate buffer and further treated as described above.

d) *Precipitation with trichloracetic acid.* The clear fluid obtained after procedure II c was acidified to pH 3—4 with N HCl, and trichloracetic acid added in substance or in 20 % solution to give a final concentration of 10 %. The precipitate formed containing the active agent was centrifuged off, washed twice in acetone, twice in ether and dried by air.

By this procedure of precipitation with trichloracetic acid a selective precipitation was apparently obtained, the preparations always being highly active.

The activity of the preparations.

The crude preparations from the pyloric mucosa of cats always showed a high activity. The average yield calculated from ten preparations amounted to about one secretory unit per 6 mg of dry substance.

Precipitation of the HCl-extracts from the pyloric mucosa of pigs with 20 per cent sodium chloride proved a very reliable procedure. Intravenous injection of an amount of dried precipitate, corresponding to one-fifth of the pyloric mucosa from one stomach caused an abundant secretion of gastric juice. This was the case whether the mucosa was minced or only roughly cut into pieces before the extraction with HCl. In the latter case however smaller amounts of inert material were extracted. Calculated in secretory units extractions of minced mucosa yielded on an average one unit per 155 mg of dried NaCl-precipitate, while those of only roughly cut mucosa gave one unit to about 50 mg.

The increase of the activity of the preparations during the different stages of purification is illustrated in table 1. As seen the activity rose successively to one secretory unit per 2—5 mg of the dry material, indicating that more than 95 per cent of the inert substances was removed. Usually only slight losses of active material occurred during purification. A comparison with the activity of a commercial secretin preparation considered to be of a high degree of purity[1] showed that 3—5 mg of this substance must be injected intravenously to give a reliable pancreatic secretion in a cat.

Numerous other ways of isolating the pyloric agent were tried. The active principle could be precipitated isoelectrically, with

[1] Pancreotest, Astra.

Table I.

The secretory activity of the preparations at different stages of purification.

Active material. (from pig)	Activity in mg dry substance per secretory unit.
NaCl-precipitate (mucosa only roughly cut in pieces before HCl-extraction)	45
I. The tannic acid method:	
a) dried residue after tannic acid precipitation	18.6
b) dried residue washed with 80 % acid alcohol	3.7
c) dried residue after precipitation at pH 8.0 and precipitation with trichloracetic acid	2.0
II. The trichloracetic acid method:	
a) dried residue after the first trichloracetic acid precipitation	5.6
b) dried residue washed with 80 % acid alcohol	4.3
c) dried residue after precipitation at pH 8.0 and precipitation with trichloracetic acid	2.3

metaphosphoric acid, phosphor-tungstic acid and 80 per cent acid alcohol. Most of the procedures tried caused no selective precipitation however. Now and then preparations of high purity were obtained but in these cases the loss of activity was too high to allow the procedure to be used as a routine method.

No furter studies of the chemical properties of the active principle were made. The contamination by inert material was too high to permit valid conclusions regarding the molecular structure. Apparently it is of protein nature and, as discussed later in this paper, shows chemical and physiological properties similar to secretin but differs from this hormone in other respects.

Physiological properties of the pyloric principle.

The secretory response to purified substance.

The gastric secretion induced by crude pyloric preparations from cat, dog and pig has been previously studied in this laboratory (Uvnäs 1942, 1943). The extracts were, in those experiments too, administered slowly intravenously to cats. The volume, acidity and peptic activity were determined. The secretory response to purified preparations did not differ in principle

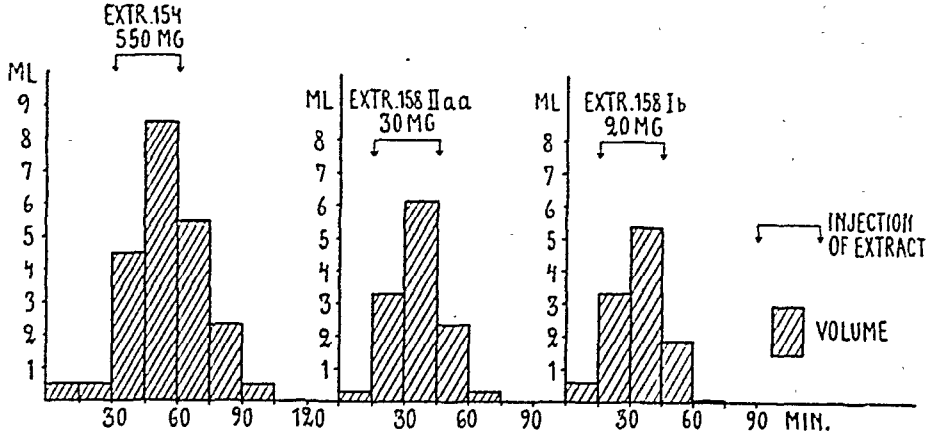

Fig. 1. Gastric secretion after slow intravenous injection of three different preparations from the pig's pyloric mucosa.
a) Cat 3.1 kg. A crude NaCl-precipitate. 550 mg injected.
b) Cat 3.0 kg. A preparation purified by the tannic acid method. 30 mg injected.
c) Cat 3.5 kg. A preparation purified by the trichloracetic acid method. 20 mg injected.

from that of crude extracts. The secretion usually began about 5 minutes after starting the injection, then gradually increased for 5—10 minutes, and then proceeded at a constant rate. After the injection was stopped the secretion continued for 10—15 minutes at maximal rate, then usually declined, reaching the basic level in 30 minutes after the end of the injection. The gastric juice was always strongly acid, the total acidity usually exceeding 150 milli equivalents per litre. The peptic power of the juice declined during the course of the secretion to very low values indicating that the excitatory principle strongly activated only the HCl-glands. A more detailed study concerning the peptic secretion will be published later.

Fig. 1 a, b and c show typical secretory responses to the injection of a crude NaCl-precipitate and two purified preparations from the pyloric mucosa of pigs. 550 mg of a crude NaCl-precipitate was suspended in 30 ml saline made slightly acid to Congo with N/HCl. After continuous stirring for 15 minutes at 40° C, it was centrifuged and the clear fluid slowly injected into the iliac vein for 30 minutes. A copious secretion of strongly acid juice amounting to 20.8 ml in 60 minutes was evoked (fig. 1 a). The undissolved residue showed no secretory potency when further extracted with acid saline. In fig. 1 b, 30 mg of a preparation purified by the tannic acid method was dissolved in

15 ml of acid saline, and injected intravenously in the usual way. 12.2 ml gastric juice was secreted in 60 minutes. Fig. 1 c represents the secretion after the injection of 20 mg of a preparation obtained by the trichloracetic acid method. 10.6 ml gastric juice was secreted in 60 minutes.

As previously observed in this laboratory (UVNÄS 1943) the secretory response to crude preparations frequently declined during the course of an experiment. Sometimes this was also seen when purified substances from pigs were used. Except for this inhibitory effect on the gastric secretion no toxic influences whatever could be observed. Peculiarly enough the inhibitory effect of the preparations from pigs often seemed to be selectively confined to the secretory activity induced by extracts from these species, the secretory response to extracts from the pyloric mucosa of cats being still unchanged. We do not know the reason for this inhibitory effect. Possibly it is due to some specific inhibitory substance (enterogastrone?) or to some desentisitation process induced by protein impurities in the preparations.

On the specificity of the pyloric principle.

Effect on pancreatic secretion. Precipitation of HCl-extracts from duodenal mucosa with 20 % NaCl and 5 % trichloracetic acid according to (IVY and others 1930) and others gives a high yield of secretin. As this hormone occurs in smaller amounts also in the pyloric mucosa our crude preparations were expected to induce a slight pancreatic secretion as well as the gastric secretion. Our expectation was fulfilled. A slight pancreatic secretion was evoked by intravenous injection of some specimens of the crude preparations from the pyloric mucosa of cats as well as pigs. However, a secretory response of the pancreatic gland was by no means always found. That the pancreatic secretion was not induced by the gastric secretory excitant was further indicated by the fact that the pancreatic excitant differed from the gastric excitant as regards its solubility in ethyl alcohol. The two experiments representated in table II A and B illustrate this fact. The flow of pancreatic juice from the cannulated pancreatic duct was registered by an electrical drop recorder. Intravenous injection of two crude pyloric preparations — one from the cat and one from the pig — both induced an abundant secretion of gastric juice and a slight pancreatic secretion. Crude

Table II.

Gastric and pancreatic secretion after intravenous administration of crude, alcohol-washed or purified substances from the pyloric mucosa of cats and pigs.

	Active material. Stage of purification	mg	Gastric secretion ml	Pancreatic secretion drops
A.	A crude trichloracetic acid precipitate from cat	75	12	11
	The trichloracetic acid precipitate from the above washed with 80 % acid alcohol	65	10	0
B.	A preparation from pig precipitated with 20 % NaCl and 10 % trichloracetic acid	50	9.0	5
	A preparation from pig precipitated with 20 % NaCl and washed with 80 % acid alcohol	40	7.5	0
	A preparation from pig purified by the tannic acid method	40	14	0
	Secretin	5	0	41

preparations washed with acid 80 % ethyl alcohol still activated the gastric glands. The pancreatic gland did not respond at all. It was invariably seen that alcoholic washing of the crude material removed the pancreatic stimulating agent, and no pancreatic secretion was seen when purified preparations were used. This fact is also illustrated in table II B, where the administration of 40 mg of a substance prepared from the pyloric mucosa of pigs by the tannic acid method caused a secretion of 14 ml of gastric juice. No pancreatic secretion was observed. A following intravenous injection of 5 mg of a commercial secretin preparation[1] induced an abundant flow of pancreatic juice (41 drops). No gastric secretion was evoked.

Effect on bile secretion. In these experiments a glass cannula was inserted in the common bile duct and the bile flow registered by an electrical drop recorder. The gall bladder duct was ligated to exclude the influence of gall-bladder contractions.

Some of the crude preparations from the pyloric mucosa of cats and pigs caused a slight increase of the bile flow. Purified substances, however, did not noticeably increase the bile flow, when injected in doses, which caused a copious flow of gastric juice.

[1] Pancreotest, Astra.

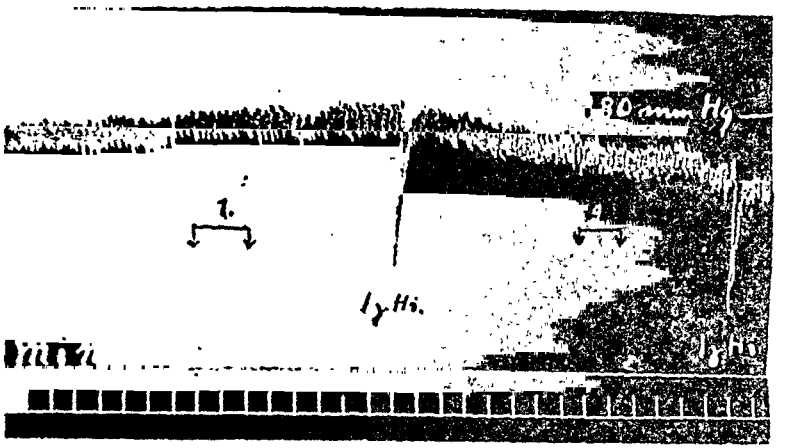

Fig. 2. Cat 3.4 kg. Blood pressure response to intravenous injection of two pyloric preparations from the pig and of histamine.

1. 50 mg of a preparation purified by the trichloracetic acid method.
2. 70 mg of a preparation purified by the tannic acid method.
1 γ histamine biphosphate causes a great fall of the blood pressure.

Effect on salivary secretion. A glass cannula was inserted in the submaxillary duct and the salivary flow electrically recorded. Salivary secretion was not seen after the administration of crude or purified pyloric preparations. Electrical stimulation of the peripheral stump of the lingual nerve before and after the administration of the preparations was always carried out as a control of the recording arrangement.

Effect on blood sugar. IVY and FISHER (1924) claimed to have obtained an insuline-like principle from the gastric and duodenal mucosa. Our active preparations did not influence the blood sugar level. The blood sugar was determined by the Crecelius-Seifert method.

Effect on gastric motility. The influence of the pyloric principle on the gastric motility was also investigated. The stomach movements were recorded by a water manometer connected with a thin rubber balloon inserted in the pyloric region of the stomach. The rubber balloon was distended by a pressure of 10—20 cm of water. No muscular movements induced by intravenous injection of crude or purified active preparations were seen.

Effect on blood pressure. It was previously observed that crude preparations from the dog (KOMAROV) or the cat (UVNÄS) did not contain significant amounts of histamine and did not in-

fluence the blood pressure, when injected intravenously. This fact could be confirmed in our purified substances. A change in blood pressure of the cat was not seen after intravenous injection of doses causing an abundant gastric secretion (see fig. 2).

Discussion.

The observations presented in this paper confirm our earlier conclusions regarding the chemical nature of the pyloric principle and are in accordance with the view of KOMAROV as presented in his recent paper (1942).

As the gastric secretory excitant is destroyed by pepsin, trypsin (duodenal juice) and is amply precipitated by NaCl, trichloracetic acid, phosphor-tungstic acid, tannic acid and other reliable protein precipitants, the protein nature of the active principle must be regarded as established. As regards chemical properties it shows great similarities to secretin. The substances are found together in the NaCl- and the trichloracetic acid precipitate. They show similarities in their solubility in some organic solvents and in their resistance to acid and alkaline solutions. However they differ in other respects, e. g. in their solubility in ethyl alcohol.

As far as has been investigated the pyloric principle selectively activates the gastric glands. No salivary, pancreatic or bile secretion is induced by the intravenous injection of doses, which evoke a copious secretion of gastric juice. The gastric juice obtained is strongly acid but poor in pepsin. These facts indicate a specificity of the gastric secretagogue as pronounced as that of secretin, which selectively activates the pancreatic gland and causes the secretion of a sodium bicarbonate solution of low enzyme content.

Strong experimental evidence favours the view that the hormonal mechanism of gastric secretion is chiefly confined to the pyloric mucosa, the secretory agent being liberated from this region (GROSS 1906, ZELIONY and ZAVITSCH 1913, KLEIN 1935, UVNÄS 1942 and many others). When in 1932 SACHS, IVY, BURGESS and VANDOLAH succeeded in isolating crystalline histamine from the pyloric mucosa this was taken to indicate that histamine was identical with the supposed gastric hormone. Yet some experimental facts argued against this theory. Histamine was

found in most of the tissues and organs of the body, and GAVIN, MCHENRY and WILSON (1933) showed that the histamine content of the corpus mucosa was even greater than that of the pyloric mucosa.

The secretory principle studied in this work is a specific gastric secretory agent predominantly localized in the pyloric mucosa. It shows no histamine-like properties but is undoubtedly of protein nature. Physiologically as well as chemically it seems to be related to secretin. All these facts strongly indicate that the active principle isolated from the pyloric mucosa is identical with the gastric hormone, *gastrin*, the excistence of which was assumed by EDKINS (1906).

Summary.

A gastric secretory excitant is isolated from the pyloric mucosa of cat and pig. The agent is of protein nature.

Two reliable methods for the preparation of the active principle in a relatively pure state are described. The methods involve the precipitation of HCl-mucosal extracts with NaCl and further precipitation with tannic acid or trichloracetic acid. Inert material is removed by washing with 80 % acid alcohol and isoelectrical precipitation at pH 8.0. Finally the active material is precipitated with trichloracetic acid.

The active agent selectively activates the gastric glands, the gastric juice obtained being strongly acid but poor in pepsin. Salivary, pancreatic or bile secretion are not influenced by the agent.

The gastric secretagogue shows physiological as well as chemical similarities to secretin. Identity with the supposed gastric hormone, gastrin, is probable.

References.

EDKINS, J. S., J. Physiol. 1906. *34.* 133.
EHRMANN, R., Int. Beitr. Path. Ther. Ernährungsstör. 1912. *3.* 382.
EMSMANN, R., Ibidem 1912. *3.* 117.
GAVIN, G., E. W. MCHENRY and M. J. WILSON, J. Physiol. 1933. *79.* 234.
GREGORY, R. and A. C. IVY, Ber. ges. Physiol. 1943. *133.* 263.
GROSS, W., Arch. Verdau. kr. 1906. *12.* 507.
IVY, A. C., J. amer. med. Ass. 1941. *117.* 1013.
—, and N. F. FISCHER, Amer. J. Physiol. 1923. *67.* 445.
—, G. KLOSTER, G. E. DREWYER and H. G. LUETH, Ibidem 1930. *95. 35.*

KEETON, R. W. and F. C. KOCH, Ibidem 1915. *37*. 481.
KLEIN, E., Arch. Surg. 1935. *30*. 162.
KOMAROV, S. A., Amer. J. Physiol. 1938. *38*. 514.
—, Rev. Canad. Biol. 1942. *1*. *191*.
—, Ibidem 1942. 1. 377.
LIM, R. K. S., C. A. IVY and J. E. MACCARTHY, Quart. J. exp. Physiol. 1925. *15*. 13.
POPIELSKI, L., Pflüg. Arch. ges. Physiol. 1909. *128*. 191.
SACHS, J. A., A. C. IVY, J. P. BURGESS and J. E. VANDOLAH, Amer. J. Physiol. 1932. *101*. 331.
SAVITSCH, V. and G. ZELIONY, Pflüg. Arch. ges. Physiol. 1913. *150*. 128.
UVNÄS, B., Acta Physiol. Scand. 1942. *4*. Suppl. XIII.
—, Ibidem 1943. *6*. 97.
—, Ibidem 1943. *6*. 117.

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Dark Adaptation and Inhalation of Carbon Monoxide.

By

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In a study McFARLAND and FORBES (1940) analyzed the effect of variations in the concentrations of oxygen on dark adaptation. A decrease in sensitivity was observed with lowered concentrations of oxygen (13.3, 11.4 and 10.0 per cent oxygen). Both the rod and the cone portions of the curves plotted from data secured with an apparatus built according to specifications outlined by HECHT and SHLAER (1938) were influenced in a similar way. These effects were counteracted by inhaling oxygen. There appeared to be a fairly close relationship between the extent of the impairment in the final rod threshold of each subject and his ability to tolerate the effects of acute anoxia as judged by his general condition.

The same authors then studied the influences of hypoglycemia on dark adaptation by injecting insulin. The thresholds were raised as soon as the effects of insulin produced a fall in the blood sugar. If afterwards the blood sugar was raised by ingesting glucose, the average threshold fell to the normal level or below it.

Considering among other things the circumstance that drivers of motor vehicles from time to time inhale appreciable quantities of carbon monoxide, it has appeared to us to be of interest to study the influence of this on dark adaptation. If a considerably lowered dark adaptation were to constitute a clear symptom of carbon monoxide intoxication, this might involve added traffic risk when driving at night if the driver were exposed to dazzle from the headlights of meeting cars.

The subjects used were persons aged about 20 years, who as far as was known had not previously been exposed to the effects of carbon monoxide or producer gas. An appropriate quantity of carbon monoxide (150—200 ml) was introduced into a KROGH's spirometer filled with air. The subjects were required to inhale this

mixture for 20 minutes. The oxygen consumed was replaced by fresh from an oxygen bomb. Immediately after the inhalation of carbon monoxide was completed the degree of saturation of the blood was determined spectrophotometrically both by HEILMEYER's method (1933) and by HAVERMANN's method (1940) both as modified by BRUNIUS. The results obtained were almost entirely in agreement. Before and after inhalation of carbon monoxide the dark adaptation of the subjects was determined. Of a total of 14 subjects, 9 were examined with an adaptometer, built in this country according to the description by HECHT and SHLAER, and 5 with biophotometer. The carbon monoxide content of the blood varied from 5—30 %. Cyanosis, dyspnoe or increase of pulse was not observed in any of the cases. One subject with 30 % carbon monoxide haemoglobin had a slight transitory headache. Otherwise there were no subjective troubles. All the subjects continued to carry out their usually work in the laboratory. These observations are entirely in agreement with what SJÖSTRAND (1942) has stated earlier.

The following results were obtained. With biophotometer determination no diminution in dark adaptation could be observed in any case. With examination by the adaptometer the values given below were obtained.

Subject	% CO-haemoglobin in the blood	Threshold-values after 30 min. dark adaptation	
		before	after
		inhalation of carbon monoxide	
1	10	3.65	3.75
2	13	3.48	3.55
3	21	3.25	3.50
4	21	3.95	4.30
5	22	3.40	3.48
6	25	3.80	4.05
7	26	3.60	4.25
8	26.5	4.10	4.10
9	30	3.60	3.60

As the threshold values on adaptation examination have been stated in logarithmic units, it is clear that in tests 4 and 7 there exists a considerable diminution of dark adaptation, in tests 3

and 6 a clear and in the remaining tests little or no diminution. There exists no parallelism between carbon monoxide content of the blood and diminution of dark adaptation.

As stated in the introduction, the threshold values of dark adaptation are influenced by the sugar of the blood. Numerous research scholars have shown that in lack of oxygen, or carbon monoxide poisoning, there arises from time to time a more or less pronounced rise of blood sugar. (See *e. g.*, ARAKI 1891, 1894, MOESCHLING 1939, VÁRADY 1942). In cases 1, 2 and 4, the blood sugar was determined before and after inhalation of carbon monoxide. The changes were minimum and could not in these cases have had any effect on the dark adaptation.

MCFARLAND and FORBES drew the conclusion from their experiments that the changes in dark adaptation observed by them must be attributed rather to influences on the visual organ nerve tissues or on the brain than on the photo-chemical processes in the retina. The experiments dealt with here would rather seem to indicate that the change of dark adaptation in question is not due to direct influence of the carbon monoxide on the restitution process in the retina, as it has not been possible to show any appreciable connection between the carbon monoxide content of the blood and the diminution in dark adaptation.

Summary.

Inhalation of carbon monoxide, producing contents of carbon monoxide haemoglobin up to 30 %, causes a diminution of dark adaptation, sometimes considerable sometimes smaller or none. It cannot be excluded that this condition may have practical significance for motorcar driving in the dark, *e. g.*, in conjunction with dazzle when meeting.

References.

ARAKI, T., Hoppe-Seyl. Z. 1891. *15.* 335; 1894. *19.* 422.
HAVERMANN, R., Klin. Wschr. 1940. *2.* 1183.
HECHT, S. and S. SHLAER, J. Opt. Soc. Amer. 1938, *28.* 269.
HEILMEYER, L., Medizinische Spektrophotometrie, Jena 1933.
MCFARLAND, R. A. and W. H. FORBES, J. gen. Physiol. 1940. *24.* 69.
MOESCHLING, Sv., Acta med. scand. 1939. *102.* 140.
—, Helvetica med. acta 1943. *10.* 39.
SJÖSTRAND, T., Socialmed. tidskr. 1942. *19.* 13.
VÁRADY, I., Wien. med. Wschr. 1942. *7.* 121.

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Method for the Determination of Oxaloacetic Acid in Biologic Systems.

By

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On account of the labile nature of oxaloacetic acid an accurate determination is difficult to obtain by the methods hitherto employed, in which the oxaloacetic acid is determined directly or through the amount of CO_2 formed by decarboxylation.

A colorimetric method for direct determination is outlined by STRAUB (1936). The principle is as follows: oxaloacetic acid forms 4-Nitrosopyrazolon-3-carbonic acid with hydrazine and nitric acid, which compound in alkaline solution has an intensive yellow colour; the concentration can then be determined colorimetrically. The method is rapid and gives the oxaloacetic acid present in amounts of 0.3 to 1.5 mg. with good accuracy.

The principle of the other methods is that oxaloacetic acid is decomposed to 1 molecule of pyruvic acid and 1 molecule of CO_2. This fact OSTERN (1933) has used for working out a manometric method with the Warburg technique. The reaction proceeds in acid acetate buffer at 5° with aniline as catalyst. EDSON (1935) has modified this method; the determination is carried out at 21°. The reaction takes place in a solution containing citric acid and aniline citrate. By these methods the values of oxaloacetic acid are found too low owing to the spontaneous decarboxylation before starting the measurement of the CO_2 formed. STRAUB (1936), too, has outlined a method for estimating oxaloacetic acid by the CO_2 formed. Here the oxaloacetic acid reacts in alcoholic solution with aniline. The CO_2 developed is absorbed in $Ba(OH)_2$ solution and determined by titration with

HCl. The accuracy is ± 6 per cent for amounts between 0.5 to 1 mg.

Common to these methods is that only the amount of oxaloacetic acid which is present at the end of the enzymatic reaction is determined. The part which is decarboxylated spontaneously is, however, not determined, hence the end result is too low. This error is considerable when the reaction takes place in acid medium.

In the following pages a method is outlined for the determination of oxaloacetic acid in enzyme experiments. The oxaloacetic acid is transformed into pyruvic acid, which is estimated together with the pyruvic acid formed spontaneously. In this way the amount of oxaloacetic acid which has not yet taken part in the enzymatic reaction is determined.

Experiments.

A. *Preparation of substrates.* Oxaloacetic acid was synthesized according to WOHL and ÖSTERLIN (1901) and pyruvic acid according to WOHL and MAAG (1910).

B. *Determination of optimal conditions for decarboxylation.* The velocity of the decomposition of oxaloacetic acid with CO_2 and pyruvic acid in dilute sulphuric acid is determined at different temperatures. The course of the transformation is measured cerimetrically. FROMAGEOT and DESNUELLE (1935) have performed a method for determination of pyruvic acid by oxydation into acetic acid by ceric sulphate and titration of the excess with ferrous ammonium sulphate. We have, however, titrated the excess of ceric sulphate iodometrically, because it is convenient to use sodium thiosulphate, the titre of which is practically constant for a long time. Unfortunately it is impossible to titrate oxaloacetic acid accurately according to this method. By titrating, 1 mol. of oxaloacetic acid is found to correspond to about 3 to 4 equivalents of oxygen, which indicates that the reaction stops at different stages. It is possible, however, to follow the decomposition, as pyruvic acid uses two equivalents of oxygen, the oxaloacetic acid thus using decreasing amounts of ceric sulphate, as the decarboxylation advances. The decarboxylation is complete when the solution consumes the amount of ceric sulphate which corresponds to the amount of pyruvic acid, which theoretically can be formed. One mol. of oxaloacetic acid (M = 132)

forms one mol. of pyruvic acid (M = 88) i. e. the pyruvic acid formed is $^2/_3$ of the oxaloacetic acid decarboxylated.

The practical procedure is as follows: in a series of tubes 1,00 ml of a solution containing about 10 mg oxaloacetic per ml is pipetted. To this is added 4 ml of H_2O + 0,5 ml dilute H_2SO_4 (6n). The tubes are placed in water-bath at a fixed temperature, at suitable intervals a tube is taken out, cooled in ice-water. Then 10 ml dilute ceric sulphate are added (FROMAGEOT and DESNUELLE 1935) and after 5 minutes the excess is titrated with 0.1-n thiosulphate. The results are given graphically in fig. 1. The abscissa is the time of reaction in minutes.

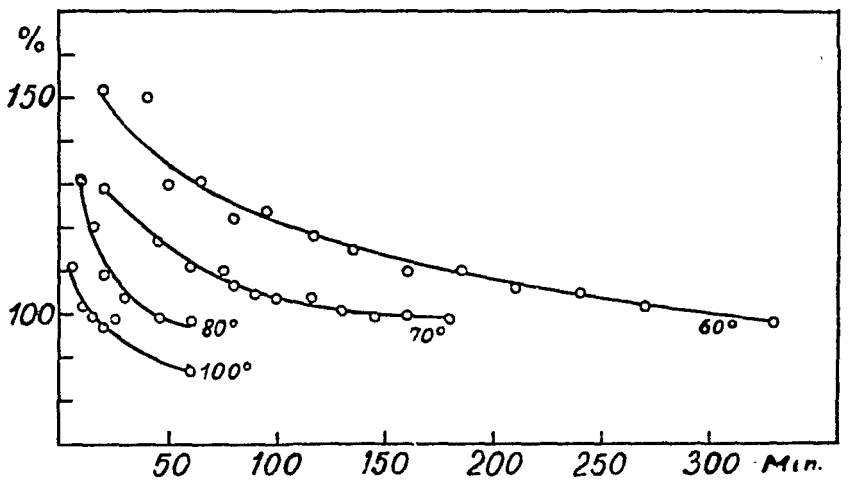

Fig. 1. The abscissa is time of reaction in minutes. The ordinate is amount of oxygen equivalents in per cent of the amount which corresponds to total decarboxylation.

The ordinate is amount of oxygen equivalents in per cent of the amount which corresponds to total decarboxylation. Simultaneously with these experiments the stability of pyruvic acid is determined under the same conditions.

It appears from fig. 1 that the most favourable temperature for decarboxylation is 70°. Here the destruction of pyruvic acid is practically zero and the space of time where the content of pyruvic acid does not deviate more than $\pm$ 1 per cent from the theoretical amount which can be formed from the initial oxaloacetic acid, is 40 min. The time of decarboxylation is 160 min. from the moment when the test has been placed in the water-bath. On account of the relatively rapid decarboxylation at 80°, viz. 40° min., this temperature might be used, although the scope

where the pyruvic acid formed only deviates 1 per cent from the theoretical value is only 10 min. In order to decide what is the most convenient, varying amounts of oxaloacetic acid were pipetted into a series of tubes to which water and dilute sulphuric acid in the above mentioned proportions were added. The tests were placed in water-bath at 80° or 70° for 40 respectively 160 min. The experimental results are given in table 1 and 2. The content of oxaloacetic acid is computed from the pyruvic acid determined analytically by multiplication with 1.5.

Table 1.

Decarboxylation at 80°.

Ox. ac. in the test mg	Ox. ac. found mg	Δ	Deviation in per cent
11.4	10.8	− 0.6	− 5.3
9.11	8.86	− 0.25	− 2.7
7.88	8.57	+ 0.49	+ 6.2
6.83	6.50	− 0.33	− 5.5
4.55	4.56	+ 0.01	+ 0.2
2.28	2.43	+ 0.15	+ 6.6
1.14	1.18	+ 0.04	+ 3.5

Table 2.

Decarboxylation at 70°.

Ox. ac. in the test mg	Ox. ac. found mg	Δ	Deviation in per cent
9.75	9.94	+ 0.19	+ 1.95
9.75	9.81	+ 0.06	+ 0.60
4.88	4.89	+ 0.01	+ 0.02
4.88	5.02	+ 0.14	+ 2.90
2.44	2.48	+ 0.04	+ 1.60
2.44	2.41	− 0.03	− 1.20
1.22	1.21	− 0.01	− 0.80
1·22	1.21	− 0.01	− 0.80

It is seen from these two series of experiments that the highest accuracy is obtained by decarboxylation at 70°. It is of great importance that the temperature should not vary more than ± 2° in order to obtain reproducable results.

B. The Analytical Procedure.

Into a volumetric flask of 10 ml 0.5 ml of dilute sulphuric acid and a suitable amount of the test is pipetted and water is added. So much test is used that the final content of oxaloacetic acid lies between 0.05 and 0.8 mg per ml. After dilution the flask is placed in the water-bath at 70° for a period of 160 min., is next cooled quickly to room temperature and the volume is brought to 10 ml by adding distilled water. To 5 ml of this mixture is added 0.5 ml 10 per cent sodium wolframate solution in order to precipitate the proteins. After filtering the content of pyruvic acid is determined colorimetrically according to STRAUB (1936).

Owing to small modifications of STRAUB's method a short description of the method used is given.

To 1 ml of deproteinized test is added 1 ml of KOH solution (100 g KOH in 60 ml water) and 0.5 ml 2 vol. per cent salicylic aldehyde in alcohol. After standing for 10 min. at 37° the test and the blank are diluted with 10 ml water. Hereby the K_2SO_4 separated is redissolved and centrifuging is thus avoided.

The colorimetric determination is then carried out with 5 mm cuvette and filter S. 47 in Pulphrich Stufenphotometer.

In table 3 the results of the analyses of oxaloacetic acid in water are recorded.

Table 3.

Determination of pure oxaloacetic acid in water.

Ox. ac. in the test mg	Ox. ac. found mg	Δ	Deviation in per cent
0.060	0.060	± 0.000	± 0.00
0.120	0.115	+ 0.005	+ 4.2
0.180	0.179	− 0.001	− 0.56
0.240	0.248	+ 0.008	+ 1.25
0.310	0.308	− 0.002	− 0.67
0.361	0.372	+ 0.011	+ 3.1
0.421	0.408	− 0.013	− 3.2
0.481	0.480	− 0.001	− 0.21
0.541	0.543	+ 0.002	+ 0.37
0.601	0.606	+ 0.005	+ 0.83

In table 4 the results of the analyses of oxaloacetic in a yeast extract not containing active carboxylase are recorded.

Table 4.

Determination of oxaloacetic acid in solutions containing yeast extract.

Ox. ac. in the test mg	Ox. ac. found mg	Δ	Deviation in per cent
0.425	0.422	− 0.003	− 0.7
0.255	0.252	− 0.003	− 1.2
0.085	0.086	+ 0.001	+ 1.2

It appears from the last column of table 3 and 4 that the accuracy of the method is satisfactory.

To get an estimate of the error which would be made, when only the amount of oxaloacetic acid which is not decarboxylated, is determined, a known amount of oxaloacetic acid is incubated with carboxylase free yeast extract in acetate buffer (pH 5.9) at 37° for one hour. At the same time the pyruvic acid formed by spontaneous decarboxylation is determined.

The results are given in table 5.

Table 5.

Determination of oxaloacetic acid in acetate buffer (pH 5.9) after incubation with yeast extract one hour at 37°.

Ox. ac. in the test mg	Ox. ac. found mg	Pyruvic acid found mg	Ox. ac. decarboxylated mg	Remaining ox. ac. mg	Remaining ox. ac.[1] mg	Remaining ox. ac. in per cent mg
0.425	0.423	0.155	0.232	0.193	[1]0.190	45.4
0.255	0.253	0.092	0.138	0.117	[1]0.110	45.8
0.085	0.084	0.040	0.060	0.025	[1]0.024	29.4

It is seen that a considerable amount of oxalacetic acid has disappeared through spontaneous decarboxylation, giving a false picture of an enzymatic reaction, when oxaloacetic acid is determined by one of the previously mentioned methods.

[1] These values are obtained by determining oxaloacetic acid according to Straub.

Discussion.

The present method has been worked out because it was necessary in some investigations carried out in this institute to determine the total amount of oxaloacetic acid, which had not interacted in the enzymatic reaction. This can of course be done by combining the pyruvic acid determination with one of those for oxaloacetic acid.

Yet it is simple and more accurate with one determination. Provided that no other substances, which reduce ceric sulphate are formed or disappear, the oxaloacetic acid can be determined as shown in B. On the other hand when alterations with regard to such compounds occur it is necessary to use specific reactions. In this case the method here described has proved to be more accurate than the methods previously outlined.

If pyruvic acid is formed or disappears during the reaction the method cannot be used. In this case oxaloacetic acid is determined according to one of the methods mentioned above.

Summary.

1) In the present paper different earlier methods for determination of oxaloacetic acid are mentioned.

2) A method for the determination of oxaloacetic acid in biologic systems with special consideration of spontaneous decarboxylation is described.

3) The accurary of the method is elucidated by experiments.

Literature.

Edson, N. L., Biochem. J. 1935, *29*, 2 083.
Fromageot, C. and P. Desnuelle, Biochem. Z. 1935, *279*, 174.
Ostern, P., Hoppe-Seyl. Z. 1933, *218*, 160.
Straub, F. B., Ibidem. 1936, *244*, 117.
Wohl, A. and Maag, Ber. dtsch. chem. Ges. 1910, *43*, 2 188.
Wohl, A. and C. Österlin, Ibidem. 1910, *34*, 1 139.

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The Cholesterol Content in Rabbit Serum.

By

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The following paper deals with the cholesterol content in rabbit serum under normal and certain experimental conditions. The order of magnitude of the individual variations and the daily variations in one and the same test animal are elucidated. A report is given of experiments showing the influence of different degrees of inanition, bleeding anaemia, ether narcosis and different surgical operations on the cholesterol content in serum. Such investigations are necessary for the evaluation of the alterations which can be demonstrated in connection with adrenalectomy and transplantation of adrenals, which will be the subject of a following paper.

Material and Technique.

In all experiments white rabbits have been used. The average weight of the animals has been about 2 kg. The animals were kept in separate cages under constant temperature. The normal diet has been kohlrabi and hay. The animals were kept on the diet for more than a week before experimentation. The animals were weighed each morning before feeding. In the diet experiments the weight of food eaten by the animal was determined very accurately. The cholesterol analyses were carried out according to BRUN's method (1935) in which both total and free cholesterol are determined. In this paper only the total cholesterol values are given. The haemoglobin concentration in blood was determined by the sicca-method (HESSE and TRIER 1937). The inaccuracy of both methods was less than 2 per cent.

Experimental.

I. Individual Variations and the Daily Variations in One and the Same Animal.

Several authors state that the serum cholesterol varies considerably in different rabbits (Sv. Hansen 1927, Grigaut 1913, Starup 1937, Teilum 1940) whereas only a few authors (Mori and Reiss 1928, Starup 1937, Teilum 1940) seem to have watched the same rabbit over a longer period. It is not possible from the works quoted above to state the magnitude of individual variations and the daily variations in one and the same rabbit, and therefore the following investigations were undertaken.

Single determinations of serum cholesterol were carried out on 211 white land rabbits. The average value was found to be 40.8 mg% with a standard deviation of 14.6. There seems to be a slight difference between male and female (male rabbit (in all 109): 35.3 $\pm$ 13.1 mg%, female rabbits (in all 102): 46.8 $\pm$ 13.8 mg%). The difference between male and female is significant; the standard deviation of the difference of the two means is 1.85.

Table I.

Daily Variations in the Serum Cholesterol Content in 12 Rabbits.

Rabbit No.	Sex	Weight in g	Total serum cholesterol in mg per cent, Day										Mean	V %
			1	2	3	4	5	6	7	8	9	10		
1	♂	2,300	27.8	31.9	31.9	32.0	36.8	32.7	35.7	35.2	35.4	26.9	32.6	10.8
2	♂	2,350	19.9	20.8	24.7	24.5	27.9	31.1	25.1	30.8	28.5	27.9	26.0	14.7
3	♀	1,800	30.7	36.1	26.6	29.8	34.7	34.4	40.1	45.3	25.1	34.3	34.7	15.0
4	♀	2,060	33.8	38.9	26.6	26.0	28.9	24.6	31.8	30.9	33.2	26.3	30.1	15.2
5	♀	1,700	24.6	32.4	35.2	32.6	28.4	27.9	32.5	35.0	26.6	22.0	29.7	14.0
7	♀	1,740	47.9	41.3	45.6	45.5	40.8	47.1	57.2	50.4	46.0	52.8	47.4	10.5
8	♀	1,960	26.2	29.6	26.5	23.1	20.4	35.4	33.8	21.0	21.7	31.0	26.4	20.8
12	♂	2,100	21.1	31.1	24.0	29.0	19.9	23.8	32.2	36.7	30.1		27.5	20.4
28	♂	1,790	31.8	32.7	34.6	30.3	32.8	32.7	44.7	55.2	52.4	52.6	40.0	25.2
29	♀	1,950	40.0	40.8	37.6	37.2	41.3	46.2	48.5	39.7	41.6	41.3	41.4	9.6
30	♀	1,830	44.4	46.0	45.4	44.5	41.3	43.8	48.5	43.5	37.2	41.7	43.5	5.8
31	♀	1,730	36.8	42.3	47.7	39.6	40.5	40.2	46.8	47.4	46.2	51.2	43.9	9.5

The results of the investigations into the daily variations in the same animal are given in detail. 12 animals were watched for a period of 10 days with one double determination per day. 3 ml. blood were taken daily.

It appears from Table I that the serum cholesterol shows variations from day to day in the normal rabbit. The magnitude of these variations is different from animal to animal and the standard deviation of the average value for the period of 10 days varies from 5.8 to 25.2 per cent. The single fractions of total cholesterol vary in the same degree as total cholesterol. The variations are of the same order of magnitude in male and female animals, and within the same litters, viz. 1, 2, 3, 4, 5 and 28, 29, 30, 31 belong to same litters.

II. The Influence of Bleeding Anaemia on the Cholesterol Content in Serum.

It is a well known fact that bleeding is followed by hypercholesterolemia (HORIUCHI 1920, BLOOR 1920, STARUP 1937). STARUP demonstrated that the hypercholesterolemia is due to oxygen deficiency, caused by the anaemia. It is of interest to find out the degree of anaemia which involves an increase in the serum cholesterol. Therefore the following experiments were carried out in which the animals were subjected to different degrees of bleeding daily.

The animals in Table I were bled 3 ml. daily without any increase in the serum cholesterol. It is seen from Table II, however, that daily loss of 6 to 15 ml. blood is followed by a hypercholesterolemia. Rabbits 72 and 74 thus show incipient hypercholesterolemia after 7 ml. blood have been drawn daily for 5 or 6 days. It should be mentioned that increase occurs when haemoglobin has fallen to 50 to 55 per cent and reaches maximum at about 30 to 35 per cent. Continued bleeding does not involve a further decrease in haemoglobin, the rabbit now being able to compensate for the loss of blood. Rabbit No. 7 has a pronounced capacity for blood regeneration, a loss of blood of 15 ml. daily being necessary to obtain a haemoglobin content of 35 per cent. The three rabbits in Table II all show incipient hypercholesterolemia at 50 to 55 per cent haemoglobin, which may be considered the limit of risk of incipient hypercholesterolemia caused by anaemia. It should be mentioned that investigations on free

Table II.

The Influence of Bleeding on Serum Cholesterol.

Day No.	Rabbit No. 72 ♂				Rabbit No. 74 ♂				Rabbit No. 7 ♀			
	Weight in g	Blood drawn in ml	Hb %	Total Chol. mg %	Weight in g	Blood drawn in ml	Hb. %	Total Chol. mg %	Weight in g	Blood drawn in ml	Hb. %	Total Chol. mg %
1	2,100	7	90	15.9	1,750	7	77	16.0	2,800	6	70	34.9
2	2,090	7	80	15.0	1,660	7	65	15.3	2,780	6	67	34.4
3	2,070	7	76	16.3	1,720	7	57	15.1	2,800	6	65	32.6
4	2,110	7	60	15.7	1,685	7	54	15.3	2,860	7	60	40.6
5	2,080	7	54	23.5	1,690	7	50	19.6	2,805	7	55	43.8
6	2,110	7	50	22.9	1,760	7	46	20.5	2,770	7	55	49.3
7	2,050	7	54	23.8	1,650	7	46	25.8	2,900	10	53	55.5
8	2,100	7	46	28.5	1,680	7	42	23.2	2,785	10	50	60.8
9	2,100	7	46	25.5	1,690	7	38	28.4	2,800	10	48	77,3
10	2,110	7	45	24.1	1,740	7	36	45.5	2,830	15	48	77.0
11	2,000	7	43	27.9	1,670	7	35	41.2	2,750	15	45	78.0
12	2,000	7	43	32.4	1,680	7	35	49.5	2,745	15	40	72.1
13	2,100	7	40	34.7	1,620	7	33	31.6	2,755	15	40	77.8
14	2,000	7	43	33.6	1,600	7	40	34.6	2,765	15	35	85.8
15	2,050	7	50	32.0	1,640	7	42	33.9	2,735	15	38	94.3
16	1,960	7	52	22.7	1,620	7	42	28.7	2,730	15	38	91.0

and esterified cholesterol showed an equal increase of both during these experiments.

III. The Influence of Different Degrees of Inanition on Serum Cholesterol.

ROTHSCHILD (1915), SHOPE (1927) a. o. have demonstrated that absolute inanition will provoke a rapidly appearing hypercholesterolemia. These results are confirmed in Table III, containing the data for three rabbits, which after a fore-period of two days do not get any food for 3 or 4 days. The increase in serum cholesterol amounts to 2 to 3 times the initial value.

Table III.

The Influence of absolute Inanition on Serum Cholesterol.

	Before inanition		During inanition			
	Days		Days			
	2	1	1	2	3	4
Rabbit No. 8.						
Totalchol. mg %	28.2	25.7	27.1	50 7	76.9	
Weight in g	2,490	2,500	2,300	2,270	2,025	
Rabbit No. 32.						
Totalchol. mg %	44.4	44.7	59.7	83.2	81.1	
Weight in g.	2,400				2,165	
Rabbit No. 33.						
Totalchol. mg %	46.6	53.1	63.2	82.6	64.9	99.9
Weight in g.	2,185	2,190	2,000	1,930	1,840	1,700

Studies on partial inanition have not previously been reported. In connection with surgical operations the appetite of the rabbits and their intake of food may be considerably decreased. It was therefore natural to examine the influence of different degrees of inanition. The results are to be found in Table IV.

This table includes 2 animals, each of which in a fore-period have got a sufficient amount of food (400 g of kohlrabi). After the fore-period rabbit No. 7 got 100 g kohlrabi for 8 days (25 per cent of the normal intake) and No. 73 200 g kohlrabi for

Tabl. IV.

The Influence of reduced Food Intake on Serum Cholesterol.

	Before food reduction				During food reduction									
	Days				Days									
	4	3	2	1	1	2	3	4	5	6	7	8	9	10
Rabbit No. 7.														
Weight in g.	2,420	2,320	2,300	2,280	2,290	2,190	2,150	2,150	2,140	2,090	2,080	2,000	1,920	
Kohlrabi eaten in g	350	395	395	375	100	100	100	100	100	100	100	100	100	
Totalchol. mg %	31.2	22.7	29.2	29.8	34.9	31.9	30.9	31.3	53 5	53.1	74.9	85.0	96.4	
Haemoglob. %	80	76	74	80	75	73	65	71	70	68	65	61	60	
Rabbit No. 73.														
Weight in g.	1,650	1,630	1,750	1,600	1,630	1,560	1,510	1,510	1,560	1,530	1,480	1,400	1,440	1,400
Kohlrabi eaten in g	400	395	400	400	200	200	200	200	200	200	200	200	200	200
Totalchol. mg. %	30.1	24.4	31.6	32.4	35.2	30.7	36.1	28.4	36.2	33.8	39.5	53.5	58.3	60.4
Haemoglob. %	81	81	81	76	68	70	72	74	68	65	63	63	60	70

9 days (50 per cent of the normal intake). In both cases a considerable hypercholesterolemia occurs, in rabbit No. 7 after 4 days and in rabbit No. 73 after 7 days. As the haemoglobin content of the blood in these animals does not decrease below 55 per cent the hypercholesterolemia must be supposed to depend upon the reduced intake of food and not upon anaemia.

IV. The Influence of Ether Narcosis and Surgical Operations on Serum Cholesterol.

Ether narcosis in man is followed by a hypercholesterolemia (MAHLER 1926, BLOOR 1914, REICHER 1908). ROTHSCHILD (1914) and LANDAU (1915) could not demonstrate a definite effect of ether narcosis on blood cholesterol in rabbits and BAUMANN and HOLLY (1923) found no change in rabbits after ether narcosis combined with different surgical operations. BAUMANN and HOLLY have only carried out a few examinations, but the author has been able to confirm their results.

In Table V are given the data for two rabbits (No. 7 and 12) which have been narcotized with ether for half an hour (35 ml. ether). No changes in serum cholesterol could be demonstrated during the days following the narcosis.

Table V also contains the results of experiments on four other rabbits. On rabbits No. 3 and No. 4 in ether narcosis 16 small pieces of psoas musculature were transplanted into the abdominal wall and on No. 9 and No. 10 a laparotomia with palpation of the intestines and adrenal regions were carried out. Neither of these procedures were followed by changes exceeding variations in normal variations in rabbit serum cholesterol.

Discussion.

The experiments in this paper form the necessary basis of investigations on the influence of the adrenal cortex on the cholesterol content in rabbit's serum. The serum cholesterol varies considerably in different rabbits and even in the same animal relatively big variations can be stated from day to day. These daily variations have not previously been reported. On a large material of white rabbits it is proved statistically, that the average value for serum cholesterol differs for the two sexes, the average value being bigger for female rabbits. This result

Table V.

The Influence of Ether Narcosis alone and combined with surgical Operations on Serum Cholesterol.

	Before narcosis			After narcosis					
	Days			Days					
	3	2	1	1	2	3	4	5	6
Rabbit No. 7.									
Weight in g.	2,505	2,450	2,435	2,360	2,410	2,380	2,385	2,330	2,350
Totalchol. mg % . .	54.6	51.2	47.6	50.7	49.8	49.1	48.9	51.2	49.0
Haemoglobin. % . . .	95	92	85	80	80	78	75	75	75
Rabbit No. 12.									
Weight in g.	2,890	2,940	2,930	2,865	2,820	2,810	2,770	2,860	
Totalchol. mg % . .	25.5	25.6	26.1	28.6	28.2	29.1	31.3	30.7	
Haemoglobin. % . . .	95	95	90	80	80	80	80	72	
Rabbit No. 3.									
Weight in g.	2,200							2,020	
Totalchol. mg % . . .	34.7	33.7	40.7	43.3	38.7	34.9	38.1	30.1	36.4
Rabbit No. 4.									
Weight in g.	2,190							2,000	
Totalchol. mg % . . .	47.2	35.7	40.6	47.7	39.4	36.2	42.5	37.6	44.3
Rabbit No. 9.									
Weight in g.	1,960							1,800	
Totalchol. mg % . . .	24.7	41.0	29.8	38.7	32.3	30.0	32.8	35.7	33.7
Rabbit No. 10.									
Weight in g.	2,130							1,940	
Totalchol. mg % . . .		25.3	28.8	29.3	28.0	21.1	29.2	22.0	20.9

is a confirmation of the results of HANSEN (1927). Daily bleeding which is followed by a decrease in blood haemoglobin below 55 per cent is followed by hypercholesterolemia, while STARUP's (1937) results seem to indicate that hypercholesterolemia does not start before the degree of 35 per cent haemoglobin. A moderate reduction of food intake, too, is followed by hypercholesterolemia.

In rabbits No. 3 and No. 4 the ether narcosis was combined with transplantation into the abdominal wall of 16 pieces of psoas musculature and in rabbits No. 9 and No. 10 with laparotomia with palpation of intestines and adrenal regions.

Both moderate anaemia and partial inanition are thus sources of error which must be taken into consideration in work dealing with the part played by adrenal cortex on serum cholesterol. Neither ether narcosis alone nor ether narcosis combined with surgical operations corresponding to extirpation and transplantations of adrenals seem to influence the serum cholesterol concentration.

Summary.

The cholesterol content has been determined in the serum of more than two hundred white land rabbits. The average value for all animals was found to be 40.8 mg per cent, the average value for the female rabbits being about 10 mg per cent higher than for male rabbits. A report is given of experiments showing variations in the same animal from day to day. Further the influence of different degrees of bleeding anaemia, inanition, ether narcosis and surgical operations is described.

References.

Baumann, E. J. and O. M. Holly, J. Biol. Chem. 1923. *55.* 457.
Bloor, W. D., Ibidem. 1914. *19.* 13.
Bloor, W. D., Ibidem. 1920. *45.* 171.
Brun, G. C., Hosp.tid. 1935, 688.
Grigaut, A., Le cycle de la cholestérinémie. Thèse, Paris 1913.
Hansen, Sv., Acta chir. scand. 1927. *62.* 483.
Hesse, H. and M. Trier, Ugeskrift f. Læger 1937, *99.* 935.
Horiuchi, Y., J. Biol. Chem. 1920. *44.* 373.
Landau, M., Die Nebennierenrinde. Jena 1915.
Mahler, A. J., J. Biol. Chem. 1926. *69.* 653.
Mori, S. and M. Reiss, Endokrinologie, 1928. *1.* 418.
Reicher, K., Z. klin. Med. 1908. *65.* 235.
Rothschild, M. A., Beitr. path. Anat. 1914, *58.* 667.
Rothschild, M. A., Ibidem. 1915. *60.* 227.
Shope, R. E., J. Biol. Chem. 1927. *75.* 101.
Starup, U., Undersögelser over experimentel Hyperlipæmi. Disp. København 1937.
Teilum, G., Om hormonal Cholesterinæmi. Disp. København 1940.

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The Selection of Food.

III. Calcium.

By

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Much information is available regarding the ability of the higher animals to satisfy their need for calcium by the choice of such food as is rich in this substance, but this information is not usually supported by objective, quantitative measurements.

Information is also available, but unchecked and lacking in detail, to the effect that children and pregnant women sometimes scrape the lime plaster off walls and eat it ("pica"); this is interpreted as an expression of a desire for calcium.

Bovine osteophagia, in the nutrition anomalies of which cattle seek and consume bones, is regarded as due to a lack of phosphates in the food. This explanation is probably correct, but it may be questioned whether there is not at the same time a desire for calcium due to its absence. However, feeding with simple calcium salts is not sufficient to remove the craving, though it seems that this may be done by feeding with pure phosphoric acid.

Under certain physiological conditions the need for calcium, and therewith the desire for it, is greatly increased. Probably the most striking example of this is the appetite of domestic fowls for material containing calcium during the egg-laying period. This question has been investigated by HELLWALL (1939), whose observations will be here reported in detail. The shell of a hen's egg weighs circa 4 g. One can therefore calculate, says HELLWALL, that a normal egg-laying hen requires during the spring 20 g of lime per week for the formation of the egg-shell alone.[1]

In HELLWALL's experiment the hens as a rule stopped laying 4 or 5 days after they were shut up in a lime-free room. The food consisted chiefly of maize, and the daily calcium yield amounted in all only to ca 0.3 g CaO. "Die Kalkhunger der Tiere nahm in diesen Tagen gerade zu groteske Formen an. Sobald der VL (Versuchsleiter) erschien,

[1] The calculation is much too high. An egg-shell can be assumed to contain 2.1 g CaO; 20 g CaO would therefore correspond to 9—10 eggs a week. As the egg-shell contains ca. 94 % calcium *carbonate*, the lime content must be recalculated according to this figure.

um zu füttern, stürtzen alle Henne, selbst die sonst sehr Scheüen, herbei, und pickten wild nach den Knöpfen seines Anzugs und nach seinen Fingernageln. Sie beruhigten sich erst, wenn er nicht mehr zu sehen war. Die Tiere schienen also die Knöpfe und Fingernägel als Kalk anzusprechen".[1] (p. 135.)

HELLWALL fed such calcium-deprived animals partly with unprepared maccaroni in pieces about 1.5 cm long, and partly with similar pieces of maccaroni filled with pulverized egg-shell and closed at the ends with dough. 5 hens received 60 pieces of prepared maccaroni and in this way consumed 3—4 g of calcium. The rest received just as many unprepared pieces of maccaroni. 4 hours later all the animals were placed "vor den Kalkhaufen". The former group now consumed only 27 g of calcium, the latter 91 g. A repetition of the experiment gave the figures 10 g and 91 g calcium respectively. The author deduces from his experiment that it is "die chemische Stimmung" in the organism and not the smell or the taste which determines the desire for calcium, for the animals swallow the maccaroni pieces whole and therefore cannot, in the author's opinion, in any way known to us recognize the presence of the calcium through the senses. Thus, the "chemical reversal" of the organism in this way arises almost immediately; a remarkable observation.

Heavy-milking cows need very much calcium. Milk contains circa 1.2 g Ca per liter. The daily production of, for example, 30 liters of milk would thus demand 36 g Ca daily, or something over 1 kg per month.

Certain horned animals which shed their horns yearly must consume great quantities of calcium while the horns are growing, and their need for calcium is comparable with that of milk cows. A reindeer's horn weighs about 3 kg. As the Ca content of the horn's bony substance very nearly agrees with that of the other bone tissues (2) it may be assumed that it contains ca 20 % Ca. Thus, for every horn-forming season ca 600 g Ca above the normal need is demanded, in so far as such considerable amounts are not mobilized from the bony system. That enormous need of Ca must be satisfied by a choice of Ca-rich food just during this period.

RICHTER (1941) and his co-workers have, in a series of publications, reported their investigation of the choice of calcium in parathyroidectomized rats. These experiments are of particularly great interest for the question under discussion.

The authors find that animals so operated chose of 2 vessels containing fluids the one containing calcium lactate and drank so much of it that the amount of calcium they obtained was sufficient for them to suppress the tetany and keep them alive. In those cases where the implantation of parathyroid tissue in the front chamber of the eye was successful the consumption of calcium lactate fell to normal figures. In 5 cases of operated rats, an increase of the basal food's calcium content resulted in their consuming only a normal quantity of the cal-

[1] A more plausible explanation is perhaps the need of harder constituents in the crop for its functioning.

cium lactate solution offered them. When parathyroid extract was injected, the "calcium appetite" did not fall to normal until the quantity per day was 100 units. A-T-10 in a quantity of 35 γ per day reduced the desire for calcium to normal figures. 3,000—8,000 I U of irradiated ergosterol, Crystalline D_2 and D_3 had the same effect. Cod-liver-oil could not be given in quantities sufficiently large to have this effect.

These experiments are extremely interesting, because they demonstrate the efforts of the organism to counteract by food selectibn the disturbance of the composition of the internal milieu due to a too low calcium content and indicated by low blood calcium figures. The author's opinion is that the choice does not depend on "a trial and error process" but that a low or high calcium content in the blood "affects all the organs of the body, including the taste buds of the tongue".

The following experimental tests to determine the desire for calcium comprise choice experiments with growing rats according to the method described in Communication I (WIDMARK, 1944).

From a large series of experiments the 2 following are reported:

Experiment I. Choice between calcium lactate and sodium lactate.
Food composition.

	g/kg
Rice flour	607
Casein	210
Dry yeast	59
Arachis oil	59
Cod-liver-oil	6
Salt mixture	59

The salt mixture was as follows:

a) the calcium-rich mixture contained the following parts per 1000:

	g/kg
Calcium lactate	356
Ferro-citrate	39
Magnesium sulphate	57
Calcium phosphate (primary)	158
Potassium phosphate (secondary)	294
Sodium phosphate (primary)	96

b) the calcium-poor mixture consisted of:

	g/kg
Natrium lactate (containing water)	472
Ferro-citrate	30
Magnesium sulphate	45
Sodium phosphate (primary)	220
Potassium phosphate (secondary)	233

The calcium content of the Ca-rich food was 15 mg per g dry food, in the Ca-poor food 2 mg. 100 g of water was added to 100 g of dry food.

Fig. 1 shows the results of the experiment.

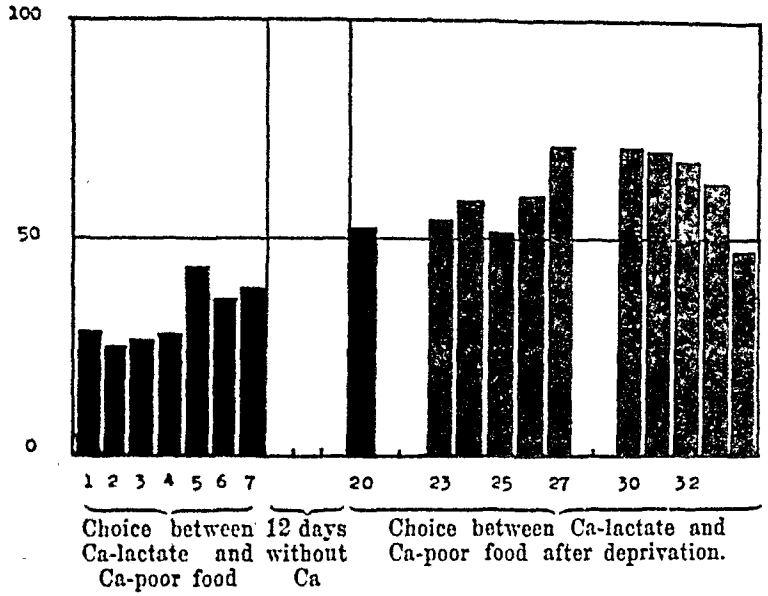

Fig. 1.

It is clear from the first measurements that the animals show almost a negative chemo-taxis for the calcium-rich food: the quota during the experiment did not exceed 43. After 12 days of the Ca-poor food the choice quota rises to above 50, and the highest value is 72.

The individual quotas, however, show great variations, which are illustrated in the following table:

No: of animal	Before Ca restriction (average of 7 days)	After Ca restriction (average of 11 days)
8	42	70
9	4	45
10	6	30
11	75	68
12	7	66
13	16	82
14	53	74
15	57	59
16	7	43
17	71	76
Average for all animals	33	61

The table shows that all the animals except one (No: 11) showed an increased calcium choice after the period of Ca-poor food. Before this period certain animals show an aversion to the calcium-rich food (6 out of 10), in one case so distinct that the quota was only 4. The average quota for all the animals before the restriction was 33, after 61.

It must be stated that in these experiments it has not been possible to give any absolutely calcium-free food in the "minus-cup" during the restriction period, because of difficulty in procuring the food and the scarcity of chemicals. Judging from the weight curves the amount in the Ca-poor food (2 mg per g dry food) was sufficiently great to not involve any slowing-down of growth worth mentioning.

A similar experiment was reported earlier (WIDMARK 1943), but in it different animals were used during a period of satisfied calcium need and after a period of lack of calcium. The experiments with calcium lactate and sodium lactate were later repeated with, in principle, the same result. Yet the casein content in the food in some of the series had to be lowered to 7.5 %, because the casein preparation at our disposal was contaminated by calcium to a fairly high degree.

Experiment II. The choice between calcium-rich and calcium-poor food, in which the calcium lactate and the sodium lactate were replaced by calcium citrate and sodium citrate equal in weight to the lactate.

In these foods the calcium content was ca 23 mg and 3 mg Ca respectively per g of dry food.

6 male and 4 female young rats were used, which at the beginning of the experiment were about 45 days old. Weight 50—60 g. After weaning they had been fed on a complete food with plenty of skim milk.

After a week's observation the animals were placed on Ca-poor food for 26 days, after which they were allowed to choose between the Ca- rich and the Ca- poor food. The results appear from the following table: this gives the average figures of the quotas for each day.

Test day	Ca
1	17
2	45
3	46
4	—
5	—
6	22
7	30
8—33	Ca-poor food
34	68
35	63
36	75
37	54
38	74
39	—
40	—
41	66
42	81
43	76
44	77
45	49
46	—
47	—
48	55
49	49
50	63

The increase in the quotas after Ca deprivation is in principle of the same nature as in the previous experiment. If the average quota figures for each animal before and after the deprivation are compared the following figures are obtained:

Experimental animal	Before Ca deprivation	After Ca deprivation
24	47	67
25	54	78
26	50	17
27	7	65
28	14	66
29	40	67
30	6	88
31	20	63
32	36	77
33	30	70

All the animals except No. 26 (male) show increased quotas after the Ca deprivation. Their average values are considerably over 50, while the quotas before the deprivation (except 25 with $Q = 54$, and 26 with $Q = 50$) were considerably under 50. Rat No. 26 differed

from the others in that it had high quotas (100, 53, 48) only the first 3 days after the Ca deprivation. After that it practically refused to eat calcium.

In this experiment the last-quoted values after the Ca deprivation showed a certain tendency to fall, which can be interpreted as the effects of a satisfied Ca need.

The experiments reported here slow clearly that an increase of the need for calcium due to a period of Ca-deprivation involves the choice by the animal of the food which is richest in calcium. The previous aversion to the calcium salts placed in the "+ cup" disappears and such a strong desire for calcium arises that the animals overcome this aversion and the chemotaxis is transformed from a negative to a high degree of positive.

In this it does not seem to be of essential importance whether the calcium is mainly a lactate or a citrate (and primary phosphate). If the taste was here the guide in the choice of the calcium-rich food one would be first compelled to assume that the calcium ion and not the salt, as such, is responsible for the positive chemotaxis. To decide this question, however, it is necessary to experiment with better defined foods, which for the present are difficult to obtain.

Summary.

1) Fully-nourished rats which had received plenty of calcium while growing (skim milk) show a negative chemo-taxis for food which contains calcium in the form of lactate and citrate.

2) On the other hand, if the animals are subjected to a period of calcium deprivation the negative chemo-taxis is transformed into positive. The animals now, for some time, select predominantly the calcium-rich food.

References.

HELLWALD, H., Z. Psychol. Physiol. Sinnesorg. 1931. *123.* 94.
RICHTER, C. P., and J. R. BIRMINGHAM, Endocrinology 1941. *29.* 655.
WEISKE, E., Landwirtschftl. Versuchsanst. 1915. *20.* 35.
WIDMARK, E. M. P., K. Lantbruksakademiens tidskr. 1943. *83.* no. 2.
WIDMARK, E. M. P., Acta physiol. scand. 1944. *7.*

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Wirkung von Arsen auf respiratorischer Gaswechsel und Blutstrom beim Menschen.

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Die bisweilen auftretende Gewichtszunahme nach längerer Verabreichung kleiner Arsendosen hat man vielfach in der Weise zu deuten versucht, dass niedrige Arsenkonzentrationen eine Hemmung besonders der oxydativen Abbauprozesse bewirken sollen, sodass die assimilatorischen Vorgänge gegenüber den dissimilatorischen überwiegen (vgl. HEFFTER und KEESER 1927). Eine Stütze hat diese Auffassung durch zahlreiche Beobachtungen an überlebenden Geweben und Zellen erhalten. Nachdem ONAKA (1911) eine Abnahme des Sauerstoffverbrauchs der kernhaltigen Gänseerythrozyten nach Zusatz von Arsentrioxyd in einer Konzentration von 0.003 % oder darüber beobachtet hatte, fand DRESEL (1926, 1927) am Rattengewebe eine ähnliche Wirkung, während die anaerobe Gärung weniger beeinflusst wurde. Bei Hefe nahmen sowohl Sauerstoffverbrauch wie Gärung unter dem Einfluss der kleinen Konzentration arseniger Säure ab. Mit Kaninchenleber erhielt KANAZAWA (1927) ebenfalls Herabsetzung des Sauerstoffverbrauchs durch Zusatz von Arsentrioxyd von 0.0001 % an. Zu ähnlichen Ergebnissen kam HORI (1929) an Leber- und Nierengewebe sowie Skelettmuskulatur und Sarkomgewebe des Kaninchens, bzw. Lymphdrüsen des Rindes. Während Hemmung regelmässig nachgewiesen werden konnte, wurde eine fördernde Wirkung der arsenigen Säure auf den Sauerstoffverbrauch nie gefunden. SZENT-GYÖRGYI (1930) sah ebenfalls Hemmung des Sauerstoffverbrauchs des Leberbreies, wenn Arsenit in einer Kon-

zentration von 0.00004 mol oder mehr anwesend war. Über den entsprechenden Effekt kleiner Konzentrationen (N/1000—N/4000) zyklischer Arsenverbindungen an Leber, Nieren-, Hoden- und Sarkomgeweben von Ratten sowie an Hefe berichteten VOEGTLIN, ROSENTHAL und JOHNSON (1931). Unter Verwendung der Nitroreduktionsmethode von Lipschitz fanden LENDLE und REINHARDT (1931) bei Froschmuskeln eine Herabsetzung der Gewebsatmung bei 0.003 % der arsenigen Säure, bei langdauernder Einwirkung auf Hefe schon bei etwa 0.002 %, und bei Froschnerven beobachteten SCHMITT, SKOW und BUEKER (1933) eine kleine, aber deutliche Abnahme des Sauerstoffverbrauchs bei einer Arsenkonzentration von 0.00002 mol. Weniger eindeutig waren die Resultate von LEIFERT (1933), der mit zerschnittener Froschmuskulatur, bzw Herzmuskulatur oder Hirnsubstanz von Säugern arbeitete. In gewissen Fällen stellte sich aber eine Hemmung des Sauerstoffverbrauchs schon bei sehr erheblicher Verdünnung des Arsenits, bzw des Arseniats (10^{-9}—10^{-8}) heraus. Spätere Versuche von SZENT-GYÖRGYI und Mitarbeitern (1935) führten zu dem Ergebnis, dass Arsenik in den Mechanismus der normalen Gewebsatmung störend eingreift, indem die Reduktion der Oxalessigsäure zu Fumarsäure verhindert wird. HUSZÁK (1935) brachte bei Kaninchen den Nachweis, dass diese Hemmung auch im lebenden Tier durch Arsen hervorgerufen werden kann und zwar durch Dosen von 3 mg pro Kg Körpergewicht, d. h. Mengen, die nur $^1/_3$—$^1/_2$ der tödlichen betrugen. Dieser Befund ist von OELKERS und VINCKE (1936) bestätigt worden. Sie erhielten ebenfalls nach 0.0002—0.0025 % Arsenik eine Abnahme des Sauerstoffverbrauchs von Leberschnitten von Meerschweinchen. Aus den Versuchen von OELKERS (1936—1937) geht ferner hervor, dass auch gewisse Fermente, wie Kathepsin sowie verschiedene Lipasen in ihrer Wirksamkeit durch Kalium arsenicosum gehemmt werden. Bereits 0.0001 % bewirkte merkliche Verlangsamung der enzymatischen Spaltungen. Man hat die Beeinflussung der Fermentwirkungen durch Arsenik mit einer chemischen Reaktion zwischen diesem und den SH-Gruppen des Glutathions, bzw des Cysteins, in Beziehung gebracht (VOEGTLIN, DYER und LEONARD 1923, LABES 1929).

Gegen den Hintergrund der erwähnten Ergebnisse erhebt sich die Frage, ob die Arsenmedikation auch den Ruhestoffwechsel des Gesamtorganismus in entsprechender Weise beeinflusst. Zwar sind die bei der therapeutischen Benutzung des Arsens im Körper

entstehenden Konzentrationen dieses Stoffes zweifelsohne weit niedriger als diejenigen, die sich in vitro als wirksam erwiesen haben, die Möglichkeit einer ungleichmässigen Verteilung muss aber auch in Betracht gezogen werden. Obgleich schon mehrere Untersuchungen hierüber vorliegen, ist eine definitive Klärung nicht erhalten worden.

BORNSTEIN und PROST (1921) experimentierten mit drei Hunden, die an Respirationsversuchen trainiert waren, und fanden nach ziemlich erheblichen Arsendosen (0.01—0.075 g) entweder ein Gleichbleiben des Grundumsatzes oder während akuter Vergiftung ein sukzessives Steigen des Sauerstoffverbrauches. NISHIURA (1924) sah bei Ratten nach 0.005— 1 mg arsenige Säure fast immer eine Erhöhung des Gasstoffwechsels, nur in einem Falle trat nach mehrtägigen Einspritzungen von 0.05 mg eine Abnahme des Sauerstoffverbrauchs ein. Bei thyradengefütterten Ratten dagegen wurde bisweilen durch sehr geringe Dosen (0.00005—0.0005 mg) eine Herabsetzung des Sauerstoffverbrauchs beobachtet (HILDEBRANDT und NISHIURA 1924). KANAZAWA (1927) bestimmte bei drei Kaninchen den Ruhegaswechsel und injizierte subkutan bei zwei der Tiere 0.005, bei dem dritten 0.003 g arsenige Säure subkutan. In den beiden erstgenannten Fällen hatte der Sauerstoffverbrauch nach 3 Stunden um 18 % abgenommen und blieb bei dem einzigen später untersuchten Tier noch nach 48 Stunden niedrig, bei dem dritten wurde $2^1/_2$ Stunden nach der Injektion eine Senkung von 29 % beobachtet, die aber später zurückging. Leider wird aber die Beweiskraft dieser Beobachtungen wesentlich dadurch beeinträchtigt, dass es unsicher erscheint, ob wirkliche Ruhebedingungen vom Anfang des Versuchs vorhanden waren. Die Bestimmungen wurden am tracheotomierten, nicht narkotisierten Tier vorgenommen, so dass eine allmähliche Abnahme des Stoffwechsels wahrscheinlich ist. Es wurde nur ein einziger Ruheversuch vor der Injektion vorgenommen, so dass die spontanen Schwankungen des Sauerstoffverbrauchs unbekannt sind. Dieselbe Anmerkung muss auch gegen die entsprechenden Versuche von HORI (1929) gemacht werden, die ebenfalls eine Senkung des Stoffwechsels nach der Arsenzufuhr zeigten. Auffallend ist auch, dass HORI in zwei von drei anämisierten Kaninchen nach derselben Dose von Arsen wie in den soeben angeführten Versuchen keine Einwirkung auf den Gaswechsel sah; in diesen Fällen war aber der Sauerstoffverbrauch schon von Anfang niedriger als in den übrigen.

Was Bestimmungen an Menschen betrifft, fand HENIUS (1902) keine Änderung des Gaswechsels ausserhalb der Fehlergrensen der Methode nach Atoxyl. BORNSTEIN und PROST stellten an drei Versuchspersonen während bzw. 17, 22 und 58 Tage durch tägliche Gaswechselbestimmungen den Einfluss mässiger therapeutischer Arsendosen fest. Sie sahen keine Wirkung des Arsens auf den Sauerstoffverbrauch — die bei zwei der Versuchspersonen eintretende kleine Erhöhung lag innerhalb der Fehlergrenzen. Zu ganz anderen Schlussfolgerungen gelangten LIEBESNY und VOGL (1923). Bei fünf von sechs Versuchspersonen mit normalem Stoffwechsel beobachteten sie »eine sehr geringe Tendenz zur Herabsetzung des Gaswechsels im Laufe der Arsenkur«, bei dem sechsten trat eine entsprechende Erhöhung des relativen Ruhewertes ein. Es handelt sich durchaus um ganz vereinzelte Bestimmungen, und die Unterschiede sind nicht grösser, als man sie von einem Tage zum anderen oft bei Gesunden findet, so dass die Beobachtungen nicht als beweisend betrachtet werden können. Ähnlich liegen die Verhältnisse bei zwei von LIEBESNY und VOGL untersuchten Fällen von Anämie. In drei Fällen von Hyperthyreose wurde eine ziemlich erhebliche Abnahme des Sauerstoffverbrauchs im Laufe der Arsentherapie notiert, in einem vierten trat zuerst eine Senkung, dann aber eine sehr grosse Steigerung ein und in einem fünften Fall mit fraglicher Hyperthyreose waren die Ergebnisse schwankend. Mit Rücksicht auf die grosse Labilität des Stoffwechsels bei Patienten mit Hyperthyreose sind die Ergebnisse meiner Meinung nach in keiner Weise überzeugend. Endlich hat KNELL (1936) die Wirkung des Arsens auf den Sauerstoffverbrauch während Ruhebedingungen sowohl in Kurzversuchen, als auch in Langversuchen geprüft. In den Kurzversuchen wurde der Grundumsatz teils unmittelbar vor, teils $^1/_2$ Stunde, 1 Stunde und 2 Stunden nach einmaliger Injektion einer geeigneten Arsenlösung festgestellt. Von den 11 Versuchen zeigten zwei eine Erhöhung des Sauerstoffverbrauchs (maximal 3—4 %) nach der Arsenzufuhr, in den anderen dagegen trat eine Abnahme ein, die maximal 1—16 % betrug; im Durchschnitt der 11 Versuche fand man eine Senkung von 5.7 %. Man vermisst in dieser Arbeit die notwendigen Kontrollversuche mit Einspritzung einer indifferenten Flüssigkeit statt des Arsenpräparates, wodurch die Bedeutung der verlängerten Ruhepause festgestellt werden könnte. Bei den Langversuchen (10—14 Tage) erhielt der Patient 10 Tage lang eine bestimmte Menge des Arsens. Über das Ergebnis teilt die

Verfasserin folgendes mit: »Die Grundumsatzwerte — — — sind nicht gleichartig, die einzelnen Ausschläge gering und liegen durchweg innerhalb der Fehlergrenzen.«

Es dürfte aus der gegebenen Übersicht hervorgehen, dass die bis jetzt ausgeführten Bestimmungen über die Beeinflussung des Gesamtgaswechsels durch Arsen keinen klaren Bescheid geben. Es lässt sich z. Z. keinen Schluss ziehen, inwieweit die bisweilen angetroffene kleine Herabsetzung des Sauerstoffverbrauchs durch Nebenumstände verursacht wurde oder nicht. Mit Rücksicht auf die prinzipielle Bedeutung dieser Frage wurde sie erneut untersucht.

Man hat hervorgehoben, dass Arsen vielleicht eine Vermehrung des Blutstroms durch eine früh einsetzende Tonusabnahme der Kapillaren bewirken kann (GOODMAN und GILMAN 1941). Dadurch wäre teils eine günstigere Resorption aus dem Darm, teils verbesserte Nutrition der Haut möglich. Ich habe deshalb auch das Minutenvolumen des Herzens und die Pulsfrequenz in den Kreis der Beobachtungen eingezogen.

In meinen Versuchen wurde der Einfluss des Arsentrioxyds auf Sauerstoffverbrauch und Blutstrom bei zwei männlichen und einer weiblichen gesunden Versuchsperson im Alter von 26—30 Jahren unter Standardbedingungen bestimmt. Die Versuchsperson kam morgens früh nüchtern ins Laboratorium und sass in vorsätzlicher Ruhe etwa 45 Minuten bequem in einem Liegestuhl, worauf der Sauerstoffverbrauch mit dem Spirometer von KROGH (1920) in einem Versuch von 10 Minuten Dauer ermittelt wurde. Etwa 10—15 Minuten nach der Sauerstoffbestimmung wurde die Pulsfrequenz gerechnet, dann wurde die arteriovenöse Sauerstoffdifferenz nach der Methode von GROLLMAN (1932) festgestellt (Vgl LILJESTRAND und NYLIN 1941). Ehe mit den Versuchen angefangen wurde, hatte die Versuchsperson während einiger Tage Übung in der Ausführung der Bestimmungen erhalten. Diese wurden dann gewöhnlich täglich — mit Ausnahme der Sonntage — vorgenommen, bisweilen fand ein Aufenthalt von 2—3 Tagen statt. Nachdem wir 14—17 Normalbestimmungen erhalten hatten, bekam die Versuchsperson täglich in Pillen 3 mg Arsentrioxyd während einer etwa ebenso langen Periode, und im Laufe dieser wurden entsprechende Bestimmungen ausgeführt. Von den Versuchspersonen bekam F. M. insgesamt etwa 90, M. S. ebenfalls 90 und O. S. 60 mg Arsentrioxyd. Die Medikation rief kein Unbehagen hervor. Das Körpergewicht zeigte während der ganzen Versuchsperiode nur kleine Schwankungen in beiden Richtungen. Aus den gefundenen Werten für den Sauerstoffverbrauch wurde teils das Mittel der betreffenden Periode nebst dessen mittleren Fehler in gewöhnlicher Weise berechnet, teils wurde auch unter Beachtung des täglich festgestellten Körpergewichtes und der Körperlänge sowie das Alter der Sauerstoffverbrauch nach HARRIS

und BENEDICT (1919) ermittelt. In der Tabelle 1 wird das Verhältnis zwischen dem direkt gefundenen Werte und dem nach HARRIS und BENEDICT berechneten als relativer Standardstoffwechsel bezeichnet. Auch für die arteriovenöse Sauerstoffdifferenz, das Herzminutenvolumen und die Pulsfrequenz werden die betreffenden Mittel nebst den mittleren Fehlern angegeben. Schliesslich werden die Differenzen zwischen den Werten der Normalperioden und denjenigen der Arsenperioden nebst den zugehörigen mittleren Fehlern berechnet.

In der Tabelle 1 werden die Ergebnisse mit Rücksicht auf den Sauerstoffverbrauch mitgeteilt. Der Verbrauch fällt innerhalb der normalen Werte, wie sie in der Litteratur angegeben werden. Der relative Standardstoffwechsel stellt sich unbedeutend niedriger aus als der Standard von HARRIS und BENEDICT heraus, was mit früheren Ergebnissen vollkommen übereinstimmt (Vgl LILJESTRAND und NYLIN 1943). Während der Arsenperiode ist der Gasstoffwechsel im Verhältnis zur Normalperiode unverändert. Bei zwei der Versuchspersonen sind die absoluten Mittelwerte für den Sauerstoffverbrauch identisch oder beinahe identisch, für die dritte finden wir zwar eine Erhöhung von 6.2 ml, entsprechend 2.6 %, die aber weniger ist als das Doppelte des mittleren Fehlers und also nicht statistisch sichergestellt ist. Die Mittel für den relativen Standardstoffwechsel zeigen offenbar nur kleine, innerhalb der Fehlergrenzen fallende Unterschiede. Es ergibt sich also, dass die Arsenzufuhr in diesen drei Fällen nicht die geringste Wirkung auf den Standardstoffwechsel ausgeübt hat.

Die Mittel für die Bestimmungen am Kreislauf finden sich in der Tabelle 2.

Die arterio-venöse Sauerstoffdifferenz zeigt in dem ersten Falle während der Arsenperiode eine Zunahme, die eben statistisch sicher zu sein scheint, in dem zweiten Falle herrscht gerade das Umgekehrte (d. h. eine Abnahme), während in dem dritten die Mittel beinahe identisch sind. Entsprechendes gilt für die Herzminutenvolumina. Mit Rücksicht darauf, dass diese Bestimmungen doch ziemlich subtil sind, was sich auch in den relativ höheren mittleren Fehlern spiegelt, glaube ich, kein grösseres Gewicht auf diese Unterschiede legen zu können. Nur so viel lässt sich mit Bestimmtheit sagen, dass erhebliche Änderungen des Blutstromes während der Arsenmedikation nicht eintreten, während kleinere Verschiebungen nicht unmöglich sind. Die Pulsfrequenz dagegen, die leicht mit ziemlich grosser Genauigkeit festgestellt werden kann, wird in allen drei Fällen während der Arsenbehandlung etwas erhöht. Bei der ersten Versuchsperson ist die Differenz

Tabelle 1.

Mittelwerte des Standardstoffwechsels.

Bezeichnungen: 1 = Zahl der Tage, 2 = Zahl der Tage, an welchen Bestimmungen ausgeführt wurden, 3 = O_2-Verbrauch, ml pro Min., 4 = Relativer Standardstoffwechsel in Prozent.

Versuchsperson	Normalperiode				Arsenperiode				Differenz Vorperiode—Arsenperiode	
	1	2	3	4	1	2	3	4	3	4
F. M., 30 J., 67 kg, m.	20	16	223.6 ± 1.67	95.6 ± 0.67	28	18	224.6 ± 2.12	93.4 ± 0.84	— 1.0 ± 2.7	2.2 ± 1.08
M. S., 26 J., 55 kg, w.	23	17	195.6 ± 1.77	99.7 ± 0.90	29	15	195.7 ± 2.31	99.9 ± 1.13	— 0.1 ± 2.84	— 0.2 ± 1.45
O. S., 27 J., 70 kg, m.	22	14	239.0 ± 2.5	96.3 ± 1.09	23[1]	16	245.2 ± 2.60	98.9 ± 1.08	— 6.2 ± 3.6	— 2.6 ± 1.53

Tabelle 2.

Mittelwerte der arteriovenösen Sauerstoffdifferenz, des Herzminutenvolumens und der Pulsfrequenz.

Bezeichnungen: 1 = arteriovenöse Sauerstoffdifferenz, ml pro 100 ml Blut, 2 = Herzminutenvolumen l, 3 = Pulsfrequenz pro Min. Die Zahl der Tage, an welchen Beobachtungen gemacht wurden, ist mit ganz unbedeutenden Verschiedenheiten dieselbe wie in Tabelle I.

Versuchsperson	Normalperiode			Arsenperiode			Differenz Normalperiode—Arsenperiode		
	1	2	3	1	2	3	1	2	3
F. M.	72.3 ± 1.38	3.12 ± 0.08	61.7 ± 0.54	80.2 ± 1.97	2.78 ± 0.07	62.8 ± 0.46	— 7.9 ± 2.42	0.24 ± 0.11	— 1.1 ± 0.71
M. S.	74.3 ± 1.54	2.65 ± 0.065	65.1 ± 0.59	66.8 ± 1.24	2.92 ± 0.058	69.3 ± 0.96	7.5 ± 1.97	— 0.27 ± 0.087	— 4.2 ± 1.13
O. S.	69.3 ± 0.97	3.44 ± 0.05	52.8 ± 0.56	70.2 ± 1.38	3.52 ± 0.07	55.9 ± 0.53	— 0.9 ± 1.69	— 0.08 ± 0.086	— 3.1 ± 0.77

[1] 3 Tage in der Mitte der Versuchsperiode wurde kein Arsen gegeben.

zwar statistisch nicht sichergestellt, in den beiden übrigen dagegen ist sie etwa das Vierfache des mittleren Fehlers. Diese Wirkung ist deshalb sicher. Als Ursache könnte man an eine geringe Erhöhung des Minutenvolumens denken, die wegen Unsicherheiten der Methode nicht nachgewiesen werden kann, es muss aber auch daran erinnert werden, dass man in Tierversuchen nach Verabreichung von Arsenpräpararaten eine Zunahme der Pulsfrequens beobachtet hat, deren Natur unbekannt ist.

Zusammenfassung.

Bei drei Versuchspersonen bewirkte die tägliche Verabreichung von 3 mg Arsentrioxyd im Laufe von 23—29 Tagen keine Änderung des respiratorischen Gaswechsels und auch keine sichere Änderung des Herzminutenvolumens. Dagegen trat eine kleine Erhöhung der Pulsfrequenz während Ruhebedingungen ein.

Literatur.

Annau, E., I. Banga, B. Gözsy, S. Huszák, K. Ladi, B. Straub und A. Szent-Györgyi, Hoppe-Seyl. Z. 1935. *236*. 1.
Bornstein, A., und H. Prost, Arch. Dermatol. Syphil. 1921. *129*. 159.
Dresel, K., Biochem. Z. 1926. *178*. 70.
—, Ebenda 1928. *192*. 351.
Goodman, L., und A. Gilman, The pharmacological basis of therapeutics. New York 1941.
Grollman, A., The cardiac output of man in health and disease. London 1932.
Harris. J. A., und F. G. Benedict, Publ. Carneg. Inst. 1919. No. 279.
Heffter, A., und E. Keeser, Handb. exp. Pharmakol. 1927. *3: 1*. 463.
Henius, M., Beiträge zur Arsenbehandlung der Chlorose, Inaug.-diss. Giessen 1902.
Hildebrandt, F., und S. Nishiura, Arch. exp. Path. Pharmak. 1924. *101*. 161.
Hori, I., Jap. J. med. sci. Transact. 1929. *4*. Pharmacol. *3*. 97.
Huszák, S., Hoppe-Seyl. Z. 1935. *236*. 66.
Kanazawa, I., Jap. J. med. sci. Transact. 1927. *4*. Pharmacol. *1*. 169.
Knell, E., Arch. exp. Path. Pharmak. 1936. *181*. 292.
Krogh, A., Wien. klin. Wschr. 1920. *35*. 290.
Labes, R., Arch. exp. Path. Pharmak. 1929. *181*. 148.
Leifert, A., Über den Einfluss von Arsenit und Arseniat auf die Sauerstoffzehrung überlebenden Gewebes. Inaug.-diss. Münster 1933.
Lendle, L., und E. Reinhardt, Arch. exp. Path. Pharmak. 1931. *162*. 585.
Liebesny, P., und A. Vogl, Klin. Wschr. 1923. *2*. 689.

LILJESTRAND, G., und G. NYLIN, Acta physiol. scand. 1941. *1.* 328.
LILJESTRAND, G., und G. NYLIN, Ebenda 1943. *5.* 194.
NISHIURA, S., Arch. exp. Path. Pharmak. 1924. *101.* 152.
OELKERS, H. A., Ebenda 1936. *184.* 276.
OELKERS, H. A., und E. VINCKE, Ebenda 1936. *182.* 499.
ONAKA, M., Hoppe-Seyl. Z. 1911. *70.* 433.
SCHMITT, F. O., R. K. SKOW und E. D. BUEKER, Amer. J. Physiol. 1934. *108.* 14.
SZENT-GYÖRGYI, A., Biochem. J. 1930. *24.* 1723.
VOEGTLIN, C., H. A. DYER und C. S. LEONARD, Publ. Health Rep. 1923. *38.* 1882.
VOEGTLIN, C., S. M. ROSENTHAL und J. M. JOHNSON, Ebenda 1931. *46.* 339.

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Wirkung von Arsen auf den respiratorischen Gaswechsel beim Meerschweinchen.

Von

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Eingegangen am 13. April 1944.

Die von mehreren Seiten unternommenen Versuche über die Einwirkung des Arsens auf den respiratorischen Gaswechsel des Menschen und verschiedener Säugetiere haben zu keinem eindeutigen Ergebnis geführt (Vgl LILJESTRAND 1944). Während einige Untersucher nach kleinen bis ziemlich erheblichen Dosen eine bisweilen beträchtliche Abnahme des Sauerstoffverbrauchs beobachtet haben, konnten andere nur eine Erhöhung nachweisen, die allerdings erst nach relativ grossen Arsenmengen auftrat; wurden kleinere Mengen gegeben, trat dagegen keine Änderung des Gaswechsels ein. Die Ungleichmässigkeit der Resultate ist zweifelsohne von Verschiedenheiten in der Versuchsanordnung bedingt. Besonders ist hervorzuheben, dass in denjenigen akuten Versuchen, in denen eine Abnahme des Sauerstoffverbrauchs nach Arsenzufuhr nachgewiesen werden konnte, die Ruhebedingungen beim Versuchsbeginn wahrscheinlich nicht genügend waren. Es war deshalb angezeigt, neue Versuche unter möglichst gleichmässigen Bedingungen auszuführen, und parallell mit den gleichzeitig hiermit publizierten Bestimmungen an Menschen (LILJESTRAND 1944) wurden deshalb auch Messungen der Arsenwirkung auf den Gaswechsel des Meerschweinchens ausgeführt, über die hier berichtet werden soll.

Für die Versuche wurden ausschliesslich männliche Meerschweinchen mit einem Gewicht von etwa 500 g benutzt. Die Tiere wurden mehrere Tage vor der Verwendung im Tierstall gehalten. Vor jeder Bestimmung blieb das Tier mindestens 11 Stunden ohne Futter, dagegen durfte es bis zum Versuchsbeginn nach Belieben Wasser trinken.

Der Sauerstoffverbrauch wurde mit dem von KROGH und LINDBERG (1931, 1932) angegebenen Apparat ermittelt. Das Tier befand sich demnach in einer kleinen Kammer, die in einem geschlossenen Respirationssystem eingeschaltet war. Das System enthielt ausserdem zwei mit körnigem Chlorkalzium gefüllte U-Röhren zur Absorption von Wasser und einen kleinen Behälter mit Natronkalk zur Aufnahme des Kohlendioxyds. Dann kam eine Waschflasche, die eine geeignete Lösung enthielt, durch welche kontrolliert wurde, dass der Kohlendioxyd quantitativ zurückgehalten wurde. Durch eine Seitenröhre stand das System in Verbindung mit einem kleinen, gut equilibriertem Spirometer. Endlich befand sich in dem System eine kleine Pumpe mit Ventilen, durch welche das Gasgemisch ununterbrochen in der Richtung durch die U-Röhren und das Natronkalkgefäss getrieben wurde. Die Tierkammer befand sich in einem Wasserbad, dessen Temperatur genau (mit Schwankungen von höchstens 0°.1) auf 30° konstant gehalten wurde. Das System wurde wiederholt mit Sauerstoff durchgespült, und vor dem Versuch wurde das Spirometer damit gefüllt. Der Sauerstoffverbrauch wurde direkt von dem Spirometer auf berusstem Papier registriert und in gewöhnlicher Weise auf 0°, 760 mm Hg und Trockenheit reduziert. Die Tiere wurden zuerst täglich — ohne Futter erhalten zu haben — in die Tierkammer gebracht, wo sie eine Stunde gehalten wurden. Erst nachdem sie sich in dieser Weise an die Versuchsbedingungen gewöhnt hatten, wurde mit den Bestimmungen angefangen. Auch in diesen Fällen blieben die Tiere zuerst eine Stunde vor dem eigentlichen Versuch in der Kammer. Sie verhielten sich bald vollkommen ruhig und behielten diesen Zustand während der Bestimmung. Gewöhnlich betrug diese 15 Minuten, in Ausnahmefällen wurden 10-Minuten-Perioden benutzt.

Nachdem an einem Tier Normalbestimmungen während 7—8 Tage ausgeführt worden waren, bekam es alle zwei Tage während 13—14 Tage subkutane Einspritzungen von Natriumarsenat (Na_2HAsO_4). Vier Gruppen von je 5 Tieren wurden untersucht. Die jedesmal verabreichte Arsenmenge entsprach einer Arsenquantität in den verschiedenen Gruppen von 1, 2, 4 bzw 8 mg pro Kg Körpergewicht. Zwei bis drei Tage nach Beginn der Arsenzufuhr wurde nochmals mit den Bestimmungen des Sauerstoffverbrauchs angefangen. Diese fanden dann täglich während 11—12 Tage mit Ausnahme der Sonntage statt. Für die drei ersten Gruppen zeigte das Körpergewicht nur unbedeutende Schwankungen, für die letzte dagegen trat eine beträchtliche Gewichtsabnahme ein. Drei dieser Tiere starben während der Versuchsperiode, offenbar infolge Arsenvergiftung. Sie sind deshalb in der untenstehenden Tabelle nicht mitgerechnet.

Die Körperoberfläche des Tieres wurde dem Ausdruck $c\sqrt[3]{p^2}$ ententsprechend in m^2 berechnet, wo p das Gewicht in g und die Konstante c zu 0.00089 gesetzt wurde (RUBNER 1887). Die gefundenen absoluten Werte für den Sauerstoffverbrauch wurden dann auf m^2 Körperoberfläche bezogen. Für sämtliche Versuche innerhalb einer Gruppe wurde das Mittel der betreffenden Periode nebst dessen mittlerem Fehler in gewöhnlicher Weise berechnet. Ebenso wurden die

Differenzen zwischen den Werten der Normalperioden und denjenigen der Arsenperioden nebst den zugehörigen mittleren Fehlern festgestellt.

Die Ergebnisse werden in der folgenden Tabelle mitgeteilt.

Tabelle 1.

Mittelwerte des Sauerstoffverbrauchs, ml pro Min. und m^2 Körperoberfläche.

Bezeichnungen: 1 = Zahl der Tage, 2 = Zahl der Tage, an welchen Bestimmungen ausgeführt wurden, 3 = Sauerstoffverbrauch.

Arsendose mg pro kg	Zahl der Tiere	Normalperiode			Arsenperiode			Differenz Normalperiode—Arsenperiode
		1	2	3	1	2	3	3
1	5	11	8	88.3 ± 1.52	12	10	85.4 ± 1.61	2.9 ± 2.20
2	5	11	8	87.4 ± 1.39	12	10	82.2 ± 1.67	5.2 ± 2.17
4	5	13	7	93.8 ± 1.45	14	10	96.7 ± 1.38	— 2.9 ± 1.97
8	2	13	7	81.4 ± 0.89	14	10	94.7 ± 1.16	—13.2 ± 1.47

Es ist offenbar, dass die Arsenmengen 1—4 mg pro kg Körpergewicht keine Einwirkung auf den Sauerstoffverbrauch der Tiere ausgeübt haben; die kleinen Unterschiede zwischen Normalperioden und Arsenperioden liegen vollkommen innerhalb der Versuchsfehler. Da die mittleren Fehler ziemlich klein sind, ist andererseits zu erwarten, dass auch eine mässige Herabsetzung des Gasstoffwechsels, wie sie von einigen Forschern früher angegeben wurde, erfasst werden müsste. Das Ergebnis steht also in bester Übereinstimmung mit den oben erwähnten Befunden beim Menschen und auch mit denjenigen der älteren Tierversuche, die am zuverlässigsten erscheinen. Es muss deshalb geschlossen werden, dass Arsen in den geprüften Dosen keine Herabsetzung des Sauerstoffverbrauchs ausübt.

Wenn die Arsendose auf 8 mg pro kg Körpergewicht erhöht wurde, trat eine unzweideutige Erhöhung des Sauerstoffverbrauchs ein. Dies ist offenbar die längst bekannte toxische Wirkung.

Zusammenfassung.

Der Ruhegaswechsel des Meerschweinchens wurde von Natriumarsenat, einer Zufuhr von 1—4 mg Arsen pro kg Körpergewicht alle zwei Tage während 12—14 Tage entsprechend, nicht beeinflusst. Nach 8 mg Arsen pro kg trat dagegen eine deutliche Erhöhung des Sauerstoffverbrauchs ein.

Literatur.

KROGH, M., und A.-L. LINDBERG, Ugeskr. Læg. 1931, *123*, 361.
KROGH, M., und A.-L. LINDBERG, Acta pathol. microbiol. scand. 1939, *9*, 21.
LILJESTRAND, G., Acta physiol. scand. 1944, *7*, 329.
RUBNER, M., Biologische Gesetze. Marburg 1887.

From the Biological Laboratories of Medicinalco Ltd., Copenhagen S.

On the Chemical Nature of the Thermolabile Fraction of the Reticulocyte Ripening Principle.

By

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Received 22 March 1944.

In previous papers it was shown that the reticulocyte ripening principle found in liver extracts and in plasma (PLUM 1942 a) consist of two fractions, one of which is thermostable and the other thermolabile (JACOBSEN and PLUM 1942). In liver extracts the former fraction has been identified as l-tyrosine (JACOBSEN and PLUM 1942).

The present paper deals with the chemical nature of the thermolabile fraction.

Our first experiments were carried out with liver extracts from which the thermolabile fraction was adsorbed on FULLER's earth and eluted with liquid phenol (fraction H in the paper of JACOBSEN and PLUM 1942). This procedure was, however, rather expensive and gave a considerable loss. After it was found by PLUM (1944 a, b) that gastric tissue contains the thermolabile fraction of the ripening principle in large amount, dessicated, defatted hog stomach was used as a very suitable raw material in the preparation of the thermolabile factor, which in this paper will be named *gastric factor*. In our purification experiments the gastric factor is extracted from the dried stomach with water, dialysed and, as it is only slightly soluble in water, allowed to precipitate from the dialysate concentrated in vacuum. The ripening power of the gastric factor was tested in ripening experiments with reticulocytes in the presence of 0.1 $^{0}/_{00}$ tyrosine

as described by PLUM (1942 a), and the ripening index (JACOBSEN and PLUM (1942) and PLUM (1942 b) was determined and calculated for a 1 ‰ solution of dry matter. Our experiments show that the gastric factor probably consists of at least two fractions.

Although we have not succeeded in isolating neither of the fractions in a pure state, our experiments gave evidence that one of the compounds must be closely related to the members of the purine family. The chemical nature of the other is still unknown.

Experimental.

100 g Ventriculin "Mco", the commercial brand of dessicated defatted hog stomach, are shaken with 800—900 cc distilled water during 1—2 hours. After sharp centrifugation the residue is extracted a second and third time, each with 500—600 cc distilled water.

The combined extracts, 1,200—1,300 cc, contain 1.2—1.3 per cent dry matter with a ripening index in presence of 0.1 ‰ tyrosine (as in all the following ripening experiments) of 0.14 —0.35 per 1 ‰ dry matter.

The extract is then very cautiously concentrated on a water bath under reduced pressure; the extract is treated under constant shaking in portions of 35 cc in a 2 litre round-bottomed flask for exactly two minutes. After one such treatment the volume is reduced to about 350 cc whereafter the evaporation is repeated once more in the same way so that the final volume is 100—150 cc. This procedure has two advantages: the considerable foaming which occurs does no harm and the extract has only been heated 4 minutes in all. Analyses show that the extract loses less than 5 per cent of the total pepsine activity under this procedure. The loss in ripening activity is generally larger and ranges from 36 to 0 per cent with an average of 22 per cent.

The concentrated extract is then dialysed through a parchment membrane against 1.5—2 l distilled water with 2—3 per cent phenol in order to prevent infection. After 20—36 hours dialysis, the 1.5—2 l dialysate is pale yellow and contains 4—6 g dry matter (the phenol is evaporated with the water).

In order to test the ripening activity 10—15 cc of the dialysate was shaken with equal amounts of ethyl ether in order to extract the phenol which destroys the blood corpuscles, and the

ether evaporated off in a vacuum under gentle heating. To the dialysate was then added 1 % sodium chloride and 1/10 vol. 1 ‰ tyrosine and the solution tested with reticulocytes in the usual way. The activity per 1 ‰ dry matter in the dialysate is generally 15—30 % higher than the activity of the original extract. The dialysate is then concentrated in vacuum in portions as described under the concentration of the gastric extract. The last part of the concentration is made on a water bath not over 40° until the dialysate has a volume of 40—50 cc. The concentration at times causes a considerable loss of activity: in one experiment the original dialysate had a ripening index with 0.1 ‰ tyrosine of 0.17 per 1 ‰ dry matter. After being concentrated to 17 % dry matter the index was found to be 0.11 and after a further concentration to 50 % dry matter the index was only 0.096. Even more potent preparations showed a corresponding fall in activity. When the dialysate is concentrated so far that the content of dry matter is above 10 %, the yellow-green solution is placed in the ice box. After 24—96 hours a white or slightly yellowish precipitate is formed. The more concentrated the dialysate, the more readily the precipitate is formed, but the ripening effect of the precipitate is then much smaller than in cases where the dialysate is not so much concentrated. The precipitate is centrifuged off, washed with a few cc of water, suspended in water and tested on reticulocytes in presence of 0.1 ‰ tyrosine. It contains practically all the gastric factor of the dialysate, as shown in table 1.

Table 1.

	Ripening index per 1 ‰ in presence of 0.1 ‰ tyrosine
Concentrated dialysate	0.15
precipitate	11.0
centrifugate	0.014

The yield of the precipitate is 20—40 mg per 100 g dessicated stomach.

The ripening effect of the precipitate varies considerably, the ripening index of 11 per 1 ‰ is the highest found, while the lowest is 0.34 per 1 ‰ dry matter. For 17 preparations the average is 3.2.

The activity is very labile and even drying of the precipitate in vacuum over sulphuric acid reduces the effect 50—75 %.

In one experiment we extracted the precipitate several times with methanol at room temperature. The methanol extract was evaporated in vacuum and the residue dissolved in water made slightly alkaline. The fraction insoluble in methanol was likewise dissolved in alkaline water. About two thirds of the dry matter was found soluble in methanol.

With the two fractions we made a ripening experiment as shown in table 2.

Table 2.

Effect of methanol soluble and methanol insoluble fraction of the gastric factor.

Vessel No.	Fraction not soluble in methanol ‰ dry matter	Fraction soluble in methanol ‰ dry matter	Tyrosine ‰	Ripening index found	Ripening index per 1 ‰ dry matter
1	0.58	0.00	0.2	2.52	4.35
2	0.29	0.00	0.2	1.32	4.55
3	0.145	0.00	0.2	0.73	5.02
4	0.073	0.00	0.2	0.36	4.93
5	0.00	1.11	0.2	2.38	2.14
6	0.00	0.56	0.2	1.02	1.82
7	0.00	0.28	0.2	0.52	1.84
8	0.00	0.14	0.2	0.34	2.31
9	0.29	0.56	0.2	4.21	5.03
10	0.145	0.28	0.2	2.03	4.83
11	0.073	0.14	0.2	1.00	4.78
12	0.037	0.07	0.2	0.50	4.78

From table 2 it is seen that a combination of the two fractions respectively soluble and insoluble in methanol gives a 50 % greater effect than should be expected if the effect was simply additive. It must thus be concluded that the gastric factor consists of at least two fractions with a mutually synergistic effect.

Chemical examination gave the following result: The precipitate directly from the concentrated dialysate is suspended in water and a few drops of concentrated ammonium hydroxide are added. Only a small part cannot be dissolved in ammonium hydroxide, this is centrifuged off and discarded as it has no ripening effect (and destroys the blood corpuscles). The remaining, bright yellow solution, is neutralized with acetic acid. This gives a reprecipita-

tion of the substance, which is centrifuged off, dried and dissolved in 1,500 parts of boiling water: after addition of a small portion of charcoal — not too much as the substance is adsorbed to the charcoal — the solution is filtered hot and allowed to cool very slowly. After 24—48 hours the precipitate is filtered or centrifuged off and dried at 100°. It then consists of small white needles or plates. The substance did not melt below 250°, is soluble in alkalis including ammonia and is precipitated by weak acids and diluted mineral acids. It is soluble in concentrated sulphuric acid under gentle heating and even in concentrated hydrochloric acid. It gives a red colour with diazobenzene sulphuric acid and a strong positive WEIDEL's reaction. The spectrum showed full

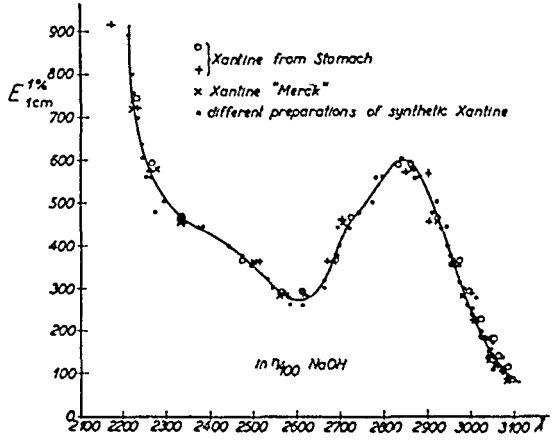


Fig. 1.

identity with that of xanthine (Fig. 1). The nitrogen content in the different preparations was found to be 31—32.5 %, calculated for $C_5H_4O_2N_4$, H_2O : 32.9 %. Spectroscopic examination of the original precipitate from the concentrated dialysate showed that about 75 % of this was xanthine.

The xanthine isolated from hog stomach has a weak, but unmistakable, effect on reticulocyte ripening, and moreover, xanthine prepared synthetically has the same effect.

This and the following experiments were carried out so that 1.0 % xanthine or xanthine derivative was dissolved in n/10 NaOH. To 0.20 cc of this solution was added 0.9 cc 2 % sodium chloride, 0.2 cc 1 ‰ tyrosine and 0.5 cc distilled water. Immediately before the addition of the blood corpuscles, 0.20 cc n/10 HCl was added. The xanthine used in this experiment was a small sample of pale yellow powder which had been stored in a sealed

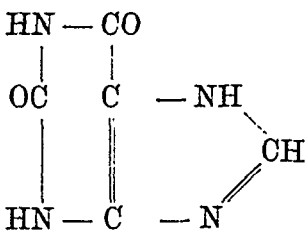
vessel for more than 30 years in the Pharmacological Institute of Copenhagen University, and was kindly supplied to us by Professor Knud Møller. This preparation showed very interesting results which shall be discussed later on. In further experiments we examined freshly prepared xanthine and a few other members of the purine group and other related substances.

Table 3.

	Monomolecular constant of ripening rate	Ripening constant	Ripening index
Saline	0.0132	—	
Standard	0.0356	0.0224	(1.00)
0.1 ‰ tyrosine	0.0150	0.0018	0.08
1 ‰ dried precipitate from dialysate of gastric extract	0.0171	0.0041	0.18
do with 0.1 ‰ tyrosine	0.0426	0.0294	1.31
1 ‰ xanthine "Merck"	0.0176	0.0044	0.20
do with 0.1 ‰ tyrosine	0.0362	0.0230	1.03

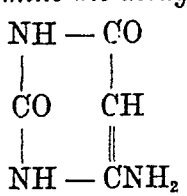
Effect of precipitate from dialysate and xanthine on the ripening of reticulocytes.

I. *Xanthine.*

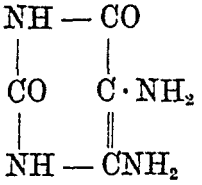


partly prepared directly and partly over guanine after the method of TAUBE (1900), II. *Guanine* prepared after TAUBE (1900), III. *uric acid*, IV. *theobromine*, V. *theophylline*, VI. *coffeine* and VII. *hypoxanthine*. Furthermore VIII. *3-methyl-xanthine* was prepared after TAUBE (1900), and the following steps in the synthesis of xanthine tested:

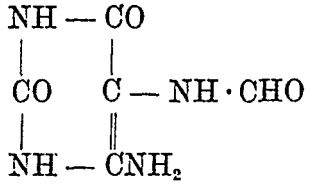
IX. *4-amino-2.6-dioxypyrimidine.*



X. *4.5-diamino-2.6-dioxypyrimidine.*

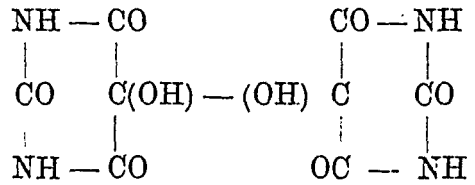


XI. *4-amino-5-formylamino-2.6-dioxypyrimidine.*

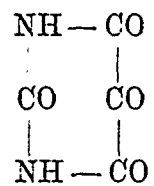


A few oxydation products of xanthine and related substances were also examined.

XII. *Alloxanthine.*

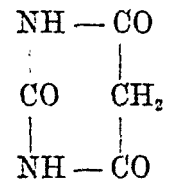


XIII. *Alloxan.*



both XI and XII being prepared from uric acid.

XIV. Finally the effect of *barbituric* acid was tested.



The results are put together in table 4.

Table 4.

No.	Name	Ripening index for 1 ‰ on presence of 0.1 ‰ tyrosine				
I.	xanthine Merck	9/4.43:	1.62	13/4.43:	1.02	1/9.43: 0.45
	xanthine prepared from urea and cyan-ethylacetate	0.40				
	xanthine from guanine	0.38	0.47	0.33	0.42	
	xanthine from stomach.....	0.35	0.40	0.37		
II.	guanine	0.08	0.07			
III.	uric acid	0.00				
IV.	theobromine	0.03				
V.	theophylline	0.10				
VI.	coffeine	0.09				
VII.	hypoxanthine	0.30				
VIII.	3-methylxanthine	0.20				
IX.	4-amino-2.6 dioxypyrimidine	0.09				
X.	4.5 diamino-2.6 dioxypyrimidine	0.05				
XI.	4-amino formylamino-2.6 dioxypyrimidine	0.59	0.69	0.82	0.34	
XII.	alloxanthine	0.22	0.24			
XIII.	alloxan	0.28	0.30			
XIV.	barbituric acid	0.04				

All substances tested have practically no effect when tyrosine is not present in the solution in the same way as described in table 3. Equally important is the fact that heating of the solution destroys the ripening effect of the purine and pyrimidine derivatives, just as it is seen with the ripening effect of the gastric factor.

The inactivation seems to be reversible and after standing the inactivated solution regains some of its activity. An example of this is shown in the following experiment: 30 mg synthetic xanthine were dissolved in 3.00 cc n/10 NaOH. A part of this solution was tested untreated and the rest was kept for 10 minutes on a boiling water bath. The heated solution was tested at once and after being kept 24 and 48 hours at room temperature.

Table 5 gives the results of the experiment.

Table 5.

No.	Blood corpuscles suspended in	Monomolecular constant of ripening rate	Ripening constant	Ripening index	Ripening index of 1 ‰ xanthine	
1	Saline	0.0139	—	—	—	—
2	standard	0.0352	0.0213	(1.00)	—	—
3	0.4 1 ‰ xanthine in n/10 NaOH 0.2 1 ‰ tyrosine 0.9 2 % NaCl 0.1 water 0.4 n/10 HCl	0.0313	0.0174	0.82	0.41	0.405
4	0.2 1 % xanthine in n/10 NaOH 0.2 1 ‰ tyrosine 0.9 2 % NaCl 0.5 water 0.2 n/10 HCl	0.0225	0.0086	0.40	0.40	
5	0.4 1 % xanthine in n/10 NaOD heated 10′ on boiling water tyrosine, saline, water, HCl as 3	0.0156	0.0017	0.08	0.04	0.05
6	0.2 1 ‰ xanthine in NaOD heated 10′ on boiling water tyrosine, saline, water HCl as 4	0.0151	0.0012	0.006	0.06	
7	saline	0.0136	—	—	—	—
8	standard	0.0338	0.0202	(1.00)	—	—
9	= 5 xanthine solution kept 24 hours at room temperature	0.0207	0.0071	0.35	0.175	0.177
10	= 6 xanthine solution kept 24 hours at room temperature	0.0172	0.0036	0.18	0.180	
11	saline	0.0142	—	—	—	—
12	standard	0.0339	0.0197	(1.00)	—	—
13	= 5 xanthine solution kept 48 hours at room temperature	0.0247	0.0105	0.53	0.215	0.26
14	= 6 xanthine solution kept 48 hours at room temperature	0.0202	0.0060	0.305	0.305	

Regeneration of the inactivated xanthine solution.

Discussion.

We have not yet been able to isolate the gastric factor in a pure form, mainly because of the extraordinary lability of the substance. This explains also the great variability in ripening power of the preparations even when they were prepared in exactly the same manner. The hitherto strongest preparation from dessicated hog stomach had a ripening index of 11 per 1 ‰ solution in presence of 0.1 ‰ tyrosine. This preparation contained 75 per cent xanthine and the xanthine thus isolated must have been present in pure form in the desiccated stomach as the procedure used cannot have caused destruction of nucleoproteins or nucleic acids. The xanthine bodies are not soluble in methanol and it is to be presumed that the ripening effect related to xanthine is to be found in the fraction insoluble in methanol.

The ripening effect of xanthine is, however, so small that the ripening effect of this fraction cannot be explained from its xanthine content. The fraction insoluble in methanol is thus not identical with xanthine, but the experiments give some evidence of a possible relation between the two substances.

Xanthine, hypoxanthine and 4-amino-5-formylamine, 2.6-dioxypyrimidine have some ripening effect; a marked, but weaker effect is found in 3-methylxanthine, alloxan and alloxanthine, but other closely related substances, guanine, uric acid, theobromine, coffeine etc. have practically no effect at all. Striking in this respect is that the guanine has no effect, but xanthine prepared from guanine by treatment with nitrous acid showed an effect equal to xanthine otherwise prepared.

The ripening effect thus seems linked to a group in the purine family. It is still more remarkable that the ripening effects of these synthetically prepared substances are destroyed by heating of the aquous solution, and that the effect is regained when the boiled solution of xanthine is allowed to stand. The synthetically prepared xanthine was recrystallized several times in boiling water or ammonia, but the dried crystals still showed an effect.

It seems probable that a ripening factor is formed from xanthine in some way. It must moreover be assumed that the ripening factor thus formed is transformed into some inactive substance. In the heat this occurs with greater velocity than xanthine forms

the gastric factor, since the ripening effect is abolished in a few minutes, while a regeneration at room temperature takes several days. When xanthine is dry it is not affected by heating to 170—180° during two hours.

In the xanthine "Merck" which had been kept in a sealed bottle for several years, the initial ripening index was much higher, but in a short time after the opening of the bottle the effect had decreased to the values found in freshly prepared xanthine. This suggested the idea that it might be possible to increase the effect of synthetical xanthine, but the results were rather uncertain; in one experiment, however, we succeeded in obtaining an increase from 0.35 to 0.52 after boiling the xanthine with acetic anhydride, washing with ether and drying at 100°.

In a single experiment I have incubated xanthine with gastric extract in order to see if the gastric extract has the power to increase the effect of xanthine. This was not the case so that if the gastric factor is formed by the influence of some enzyme in the stomach on xanthine this enzyme is not extracted with water from the dried tissue.

The chemical nature of this fraction of the gastric factor is still unknown. It may be a dehydration product of xanthine or it may as well be some desmotropic form; it is impossible to decide between these possibilities before we have succeeded in isolating a stronger preparation of the xanthine factor so that it is possible to examine its physical properties. Still more problematic is the rôle of the gastric factors in the ripening processes, how they work with the tyrosine or the tyrosine derivatives, and the experiments here described are of course unable to throw any light upon this side of the physiology of the reticulocytes ripening.

Summary.

Some attempts to isolate the thermolabile fraction of the reticulocyte ripening principle from dessicated hog stomach are described.

This gastric factor is found dialysable and when purified very little soluble in water. It is very labile. The factor can be divided into two fractions, one soluble and one insoluble in methanol. The strongest preparation from dessiccated hog stomach contains 75 % xanthine which can be isolated from the gastrix extract.

Pure synthetic xanthine has a slight ripening effect. Hypoxanthine, 3-methylxanthine, 4-amino 5 formylamino 2—6 dioxypyrimidine, alloxan and alloxanthine have the same effect. Little or no effect have guanine, uric acid, theobromine, theophylline, coffeine, 4-amino 2—6 dioxypyrimidine, 4—5 diamino 2—6 dioxypyrimidine and barbituric acid.

When xanthine is heated dry the ripening effect is not altered, but when heated in water, the effect disappears to reappear after standing.

References.

JACOBSEN, E., and C. M. PLUM, Acta physiol. scand. 1942. *4*. 272.
PLUM, C. M., Ibidem 1942a. *4*. 259.
—, Acta med. scand. 1942b. *112*. 151.
—, Undersogelser over Reticulocytmodningen in vitro. Diss. Copenhagen 1944a.
—, Acta med. scand. 1944b. (In press.)
TAUBE, W., Ber. dtsch. chem. Ges. 1900. *33*. 1371. 3043.

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On the Peptidase Activity in Papain Preparations.

By

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Various workers have brought forth evidence at to the composite nature of the proteolytic system in papain preparations. WILLSTÄTTER and GRASSMAN (1924) demonstrated that papain solutions activated with HCN digested as different substrates as gelatin, pepton and leucyl-glycyl-glycine. In a series of papers BERGMANN and coworkers (1935, 1936 a and b) studied the substrate specifity of two peptidases in papain, tentatively designated by them as Papain I and Papain II. The first one is characterized by splitting benzoylisoglutamine and hippurylamide and is completely inhibited by phenylhydrazine and other carbonyl reagents. The second peptidase splits pepton ex albumine and gelatin, and is activated by phenylhydrazine. In a more recent paper BERGMANN and ROSS (1936) suggested the possibility that there ocurred in papain a special enzyme with aminopolypeptidase activity. In enzyme solutions inactivated with H_2O_2 and then reactivated with HCN there reappeared only the gelatin splitting activity and not the aminopolypeptidase activity. As substrate peptides built up from leucine and glycine were used. In 1939 BALLS and LINEWEAVER described the properties of a crystallized enzyme preparation from papain. The enzyme hydrolyzed both hippurylamide, casein and haemoglobin. In a recent paper BALLS and JANSEN (1941) described the crystallization from papaya latex of a second enzyme closely related to the chymotrypsin of the pancreatic juice. Thus some light has been thrown

on enzymes with endopeptidase activity (proteinases) whereas the properties and especially the substrate specifity of the exopeptidases (peptidases) in papaya latex are still relatively unknown. In the present paper a series of peptides all containing free amino and carboxylic groups and which therefore could be split only by exopeptidases of the type aminopolypeptidase, were digested with several papain preparations.

Experimental.

Methods. For the determination of the exopeptidase activity the micro methods of LINDERSTRÖM-LANG and HOLTER (1931, 1932) were used. Unless otherwise stated the following substrates were digested in 0.2 M solutions at pH 5 (glass-electrode): alanyl-glycyl-glycine, alanyl-glycyl-alanine, glycyl-leucyl-glycine, alanyl-leucyl-glycine, glycyl-leucyl-glycine, leucyl-glycine, glycyl-glycine, valyl-glycine, glycyl-leucine, leucyl-alanine, valyl-alanine, alanyl-glycine, glycyl-valine, glycyl-alanine, valyl-alanine and alanyl-leucine. All the peptides were prepared according to FISCHER (1906, 1923). The purity of each peptide was controlled by determinations of total nitrogen, amino nitrogen, and melting points. In the tables the names of the peptides are abbreviated. Thus AGG stands for alanyl-glyclyl-glycine, VG for valyl-glycine LG for leucyl-glycine and so on.

Six papain preparations were used which were termed 1, 2, 3, 4, 5, 6. Preparation 1 was obtained from the British Drughouse, nr 2 was a specimen of directly dried juice from papaya fruits, nr 3, 4, 5, 6 were all from the Merck Corporation with activities given respectively as 1 : 100, 1 : 200, 1 : 200 and 1 : 300. The enzyme solutions were prepared by grinding the papain preparation to a smooth paste with a small amount of water in a mortar. The pastes were diluted (by grinding) to the volume required and then filtered through paper. 7 cmm^3 of various dilutions of these solutions were incubated in test tubes usually at 40° C with 7 cmm^3 of the substrate solution. When activated papain solutions were used, 1 ml of papain solution was incubated at pH 5.0 with 0.5 ml of a 0.5 mol solution of HCN for 2 hours at 40° C. In the digestion tests 7 cmm^3 of this activated solution were used. The digestion was interrupted by adding 150 cmm^3 of alcoholic tymolphtalein solution (tymolphtalein in 90 % alcohol). The titration of the digestion tests was carried out with n/20 $(CH_3)_4$ NOH.

The determination of the endopeptidase activity was carried out with gelatin as substrate according to the directions of WILLSTÄTTER and GRASSMAN (1924). Each ml of enzyme solution contained the material solved from 15 mg of the papain preparation. The pH of the solution was corrected to 5.0. The HCN-activation was carried out for 2 hours at 40° C with 2 ml of enzyme solution, 1 ml of citrate buffer (pH 5.0) and 1 ml of HCN-solution (pH 5.0). At the end of the activation period 6 ml of the gelatin solution were added.

Results.

The papain preparations were rather inhomogenous as shown by the values obtained when determining total nitrogen (micro-Kjeldahl) and the solubility of the six preparations. A series of typical values are given in Table 1.

Table 1.

The Determination of Total Nitrogen and Solubility of Papain Preparations.

Preparation number	Total Nitrogen in per cent	Per cent of Preparation Solved by Shaking 0.5 g with 10 ml of Water
1	3.1	15.5
2	10.0	51.0
3	3.0	14.5
4	5.7	30.5
5	11.1	55.0
6	7.5	36.5

It is evident that there is a parallelism between the solubility and amount of total nitrogen of the enzyme preparations. For the determination of the endopeptidase activity of the papain preparations gelatin was used as substrate and the digestion at first carried out according to WILLSTÄTTER and GRASSMAN. Typical results from a series of determinations are given in Table 2.

Table 2.

The Hydrolysis of Gelatin with Activated Papain Preparations. Digestion performed at 40° C and at pH 5.0 according to WILLSTÄTTER and GRASSMAN.

Digestion time in hours	The Activity in ml 0.2 N KOH in 90 percent Alcohol					
	Prep. 1	Prep. 2	Prep. 3	Prep. 4	Prep. 5	Prep. 6
1	0.30	0.60	0.50	0.40	0.50	0.35
2	0.40	0.65	0.60	0.50	0.55	0.40
3	0.45	0.70	0.70	0.60	0.60	0.50

On comparing the results given in Tables 1 and 2 it will become evident that there exists no correlation between the solubility of the preparations and their proteolytic effect. When comparing the figures in Table 2 with those obtained by WILLSTÄTTER and GRASSMAN under similar conditions it is obvious that the preparations used in the present investigation had a rather low proteolytic activity. The reason may be that the

enzyme preparations used in this investigation may have been old and therefore were inactivated. Under the present conditions no fresh enzyme preparations were available.

In order to facilitate a comparison between the endopeptidase and exopeptidase activity of the preparations, gelatin digestions were also carried out by the micro methods of LINDERTSRÖM-LANG and HOLTER. Estimations of liberated amino and carboxylic groups were made. In both the series a 6 % solution of gelatin was used. 1 ml of enzyme solution contained the material solved from 5 mg of papain preparation, and this volume of enzyme was activated with 0.5 ml 0.5 mol HCN for 2 hours at 40° C and pH 5.0. 7 cmm³ of activated enzyme solution were incubated with 7 cmm³ of substrate (gelatin) for 2 hours at 40° C and pH 5.0. In the determination of the amino groups the digestion was completed by adding a surplus of hydrochloric acid (0.2 N) and 150 cmm³ of indicator solution (naftylamine in 90 % acetone). The titrations were carried out with n/20 ammonium acetate in 90 % alcohol. The determinations of the carboxylic groups liberated during the digestion of gelatin with papain were made with the same amounts and concentrations of enzyme and substrate and during the same experimental conditions as used in the determination of the amino groups. The digestion was completed by adding 150 cmm³ of indicator solution (tymolphtalein in 90 % alcohol). The titration was made with n/20 $(CH_3)_4$ NOH. With this method of digestion the experimental conditions were the same as when determining the aminopolypeptidase activity of the papain preparations.

The results of typical serial determinations of the endopeptidase activity in preparations 5 and 6 as obtained by these methods are given in Table 3.

Table 3.

The Hydrolysis of Gelatin with Activated Papain Preparations. Digestion performed 2 hours at 40° C and at pH 5.0 according to the micro methods of LINDERSTRÖM-LANG and HOLTER. Activity in cmm n/20 NH_4COOCH_3 and $(CH_3)_4NOH$.

Preparation number	Amino groups in cmm n/20 NH_4COOCH_3	Carboxylic groups in cmm n/20 $(CH_3)_4NOH$
5	5.9	5.6
6	4.6	4.4

In determining the aminopolypeptidase activity of the papain preparations the effect of varying the relation i. e. amount of enzyme to amount of substrate was first tested. Three solutions of enzyme, containing material from 50 mg, 5 mg and 1 mg of papain preparation per ml of solution were made. 7 cmm³ of each of the three enzyme solutions were incubated with 7 cmm³

of a 0.2 mol solution of alanyl-glycyl-glycine for 2 hours at 40° C and at pH 5.0, after a preliminary activation with HCN in the same way as described above. Typical results of a series of determinations are given in Table 4.

Table 4.

Hydrolysis of Alanylglycylglycine with Different Concentrations of Activated Papain-Enzyme solutions. Enzyme Solutions prepared by Extracting 50 mg, 5 mg and 1 mg respectively with 1 ml of Water. Digestion time 2 hours at 40° C and at pH 5.0.

Preparation number	Activity in cmm n/20 $(CH_3)_4NOH$		
	50 mg prep./ml	5 mg prep./ml	1 mg prep./ml
1	1.0	3.5	1.2
2	2.1	3.4	0.5
3	2.0	3.2	0.5
4	2.2	3.6	0.8
5	1.8	4.0	1.2

In accordance with these results the enzyme solutions used in the analysis of the aminopolypeptidase activity of papain preparations were made by extracting 5 mg of papain with 1 ml of water. In Table 5 the results of a typical series of digestion experiments in which di- and tripeptides were used as substrates are given.

Table 5.

Hydrolysis of a Series of Peptides with Activated Papain Prep. nr 5; 5 mg/ml Substrate-Concentration 0.2 mol. Digestion-time 2 hours at 40° C and at pH 5.0.

Enzyme Activity in cmm n/20 $(CH_3)_4$ NOH																Substrate
AGG		AGA		GLA	ALG		GLG	LG	GG	VG	GL	LA	VA	AG	GA	AL	
2.8		2.5		1.5	2.5		3.5	0	0	1.2	0	0.6	0	1.0	0	0	Enzyme activity

On comparing with the results obtained by digesting the same series of peptides under similar experimental conditions but using an aminopolypeptidase solution prepared from the hog's pyloric mucosa (ÅGREN, 1942 b) it will become evident that aminopolypeptidase from papain and the pyloric mucosa have a similar range of substrate specifity.

Under experimental conditions as indicated in Table 5 no digestion was demonstrable when using not activated papain

solutions. This result is in accordance with the experience of GRASSMAN and WILLSTÄTTER (1924) when investigating the papain digestion of leucylglycylglycine and peptone. These authors also stated that the temperature optimum of the papain digestion of gelatin occurred at about 60° C. In some experiments it was tested whether aminopolypeptidase activity of HCN-activated papain solutions had a similar high temperature optimum. The results of a typical series of digestion experiments are given in Table 6.

Table 6.

Hydrolysis of Alanyl-glycyl-glycin by Activated Papain at 40° C and 60° C. Papain Prep. nr 5; 5 mg/ml. 0.2 mol Solution of Substrate. Digestion-time 2 hours at pH 5.0.

Preparation number	Activity in cmm n/20 $(CH_3)_4$ NOH at	
	40°	60°
5	2.5	2.2
6	3.1	2.5

Obviously the temperature optimum of the aminopolypeptidase activity is not the same as that of the enzyme digesting gelatin.

Discussion. The results of BERGMANN and coworkers (1935, 1936) and more recently of BALLS and coworkers (1939, 1941) are in accordance with the view that the juice of Carica papaya contains at least three proteolytic enzymes one of which is an aminopolypeptidase. The occurrence of this enzyme had previously been reported by WILLSTÄTTER and GRASSMAN (1924). The substrates used by these authors were built up by two amino acids, leucine and glycine. In 1935 BERGMANN, ZERVAS and FRUTON showed that the tripeptide glycyl-glutamyl-glycine was not split by papain. One of the main results of the present investigation is the demonstration of the broad substrate specifity of the aminopolypeptidase of papain (Table 5). The concentration of the aminopolypeptidase activity also seems to be of the same order of size as that of the protease activity (compare Tables 3 and 5).

With regard to substrate affinity, the parallelism between the aminopolypeptidase activity of papain preparations and that of the pyloric mucosa of hog (ÅGREN 1942 b) is specially interesting. It is likely that greater importance than one is inclined to believe, will have to be attached to this observation. The present author has previously reported experimental coincidence in favour

of the view that the aminopolypeptidase of the hog's pyloric and duodenal mucosa may be identical with CASTLE's intrinsic factor (ÅGREN, 1942 a, b; 1943). If this hypothesis is viewed in the light of the present results it may be suggested that CASTLE's intrinsic factor is not strictly bound to the mucous membrane of the alimentary canal. A factor with similar enzymatical properties may exist outside of the body. Consequently it should be possible that patients suffering from pernicious anemia could be supplied with food containing both extrinsic and intrinsic factors and that as a result of the interaction of these factors a resorbtion of the antipernicious anemia factor may take place. The result would be labelled as "spontaneous remission".

Experimental evidence supporting this theory may be taken from a paper recently published by DAVIS, DAVIDSON, RIDING and SHAW (1943). The idea occurred to these authors that it might be advantageous to administrate to patients suffering from pernicious anemia whole liver in a soluble, predigested form. Papain was chosen as a suitable enzyme for the digestion of the liver, as this enzyme was known to react at the natural pH of the minced liver — namely approximately at pH 5.6. DAVIDSON and coworkers did not add any activator to their papain solution, but an activation of the papain enzymes nevertheless may have occurred owing to the cystein and glutathion present in the liver brei. The preparation was tested on patients suffering from pernicious anemia. The authors obtained a satisfactory response in reticulocyte counts, followed by a progressive amelioration of the clinical and haematological conditions with the daily administration of doses of the "proteolyzed" liver preparation deriving from about 47 g of raw liver. Since 250 g of raw liver is the usual minimal quantity necessary to produce a satisfactory response in pernicious anemia the possibility is not to be excluded that these authors — although they have not mentioned the possibility — actually had obtained a real activation of extrinsic factor activity present in the raw liver according to the same principles as used by SJÖGREN (1940). In the English experiment the intrinsic factor may have been aminopolypeptidase present in the papain preparation.

Recently, the present author demonstrated the close relation existing between the aminopolypeptidase activity and the intrinsic factor activity of hog's pyloric and duodenal mucosa. Aminopolypeptidase from this material a hundred times purified

seems to have maintained the intrinsic factor activity of the original material i. e. the pyloric and duodenal mucosa, unimpaired. This result certainly strengthens the author's theory that there exists an identy between aminopolypeptidase and intrinsic factor. The occurrence of an enzyme with the same range of substrate specificity in papain as well as the results obtained by DAVIS and coworkers seem to support the possibility that the ntrinsic factor of CASTLE may exist outside of the body.

Summary.

It has been demonstrated that papain preparations contain an aminopolypeptidase with a substrate specificity of the same type as that of the corresponding enzyme present in the hogs pyloric and duodenal mucosa. Based on recent experimental evidence the view is advanced that the intrinsic factor of CASTLE may occur in certain vegetable material.

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References.

BALLS, A. K., and E. F. JANSEN, J. biol. Chem. 1941. *137*. 459.
BALLS, A. K., and H. LINEWEAVER, Ibidem 1939. *130*. 669.
BERGMANN, M., and W. F. ROSS, Ibidem 1935. *111*. 659.
BERGMANN, M., and L. ZERVAS, Ibidem 1936. *114*. 711.
BERGMANN, M., L. ZERVAS and J. S. FRUTON, Ibidem 1935. *111*. 221. 245.
— — —, Ibidem 1936. *115*. 593.
CASTLE, W. B., Amer. J. med. Sci. 1929. *78*. 748.
DAVIS, L. J., L. S. P. DAVIDSON, D. RIDING, and G. E. SHAW, Brit. Med. J. 1943: *1*. 655.
FISCHER, E., Untersuchungen über die Aminosäuren, Polypeptide and Proteine, Berlin 1906. I. and 1923. II.
LINDERSTRÖM-LANG, K., and H. HOLTER, Hoppe-Seyl. Z. 1931. *201*. 9.
— —, Ibidem 1932. *204*. 15.
— —, Ibidem 1934. *226*. 149.
SJÖGREN, B., Acta med. scand. 1941. *106*. 479.
WILLSTÄTTER, R., and W. GRASSMAN, Hoppe-Seyl. Z. 1924. *138*. 184.
ÅGREN, G., Enzymologia 1942. *10*. 161.
—, Ark. f. Kemi, Mineralogi, Geologi 1942. *16B*. n:o 6.
—, Ibidem 1943. *17B*. N:o 16.

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Energy Production, Pulmonary Ventilation, and Length of Steps in Well-trained Runners Working on a Treadmill.[1]

By

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Even if persons who are apparently of equal muscular strength go in for the same hard training, e. g. running, they do not, as we know, achieve the same results; a few persons distinguish themselves by outstanding achievements, and, frequently through a great number of years, they are in a class of their own. The causes of this may vary. For medium and long distances, an individual is required whose organs must be especially efficacious in several respects. Moreover, the person in question must be able to perform the work of running as economically as possible, thus transforming a comparatively great part of the energy developed into specific work. He must also be able considerably to increase the aerobic output of energy, this being a requisite of all prolonged muscular exertions. As to the latter point, it will appear from investigations by ROBINSON and his collaborators that if world-champions and other well-trained individuals, who do not actually belong to the top-class, perform the same very intensive work, the absorption of oxygen during work of the same intensity is highest in people with a world record, whilst

[1] The experiments were performed in collaboration with E. HOHWÜ CHRISTENSEN with the support of Miss P. A. Brandt's endowment and P. Carl Petersen's fund.

on the other hand they accumulate less lactic acid in the blood a fact which shows that their work is more aerobic than that of the others. The capacity of absorbing large amounts of oxygen during hard work is thus one of the characteristic features of the best long-distance runners. The experiments referred to belong to running over medium distances.

The second question: Whether the best runners also run more economically than less good runners has not been previously examined. It is true that ROBINSON and his collaborators found that world-record runners absorbed more oxygen than less good runners in performing the same very intensive work; but at the same time their determinations showed that the less good runners accumulated far more lactic acid in their blood than the world-record runners, which indicated that the former covered a larger part of the energy transformation by anaerobic energy output. Consequently, as a "steady state" has not been arrived at in this very intensive work, it cannot be concluded from the oxygen absorption measured during the work exactly how large an energy transformation it required.

One would expect the best runners to have a lower oxygen consumption when running than the less good ones, as it has been found in numerous experiments, among other things on the bicycle ergometer, that the efficiency increases as the subject becomes accustomed to the work. It cannot be taken for granted however, that a comparison of the very best runners with others of a slightly inferior class will necessarily show this, as in both cases the technique can be presumed to be very nearly perfect in an athletic exercise like running. In order to examine this problem we measured and compared the energy production in running at varying speeds on a horizontal treadmill, partly in some runners entered for the medium distances, partly in some well-trained athletes who had not gone in for running with a special view to competition.

Another problem of interest is the strain to which the organism is exposed by the different kinds of athletics. If, for instance, the metabolic increase produced by a certain form of exercise is known, we may form an estimate of the demands made on the respiratory and circulatory functions.

In this series of experiments, determinations of the metabolism and of the pulmonary ventilation were made during running at speeds from 4.05 to 18.6 kilometres per hour and walking at

speeds varying between 5.0 and 12.78 kilometres per hour — a total of 105 determinations of metabolism. All experiments were performed on the treadmill in the horizontal position. Although the results of these investigations were obtained by experiments on a treadmill and not on the sports ground, where the surface is different and the resistance of the air, especially at the higher speeds, plays a not inconsiderable part, they still give an idea of what running and walking at the speeds in question require. In view of the absence of air-resistance and the smooth surface, — a broad rubber belt, — the figures must be lower than they would be, had they been measured on the sports ground.

A number of previous investigations, especially at ordinary rates of speed, will not be referred to in this publication, in which higher speeds are chiefly studied. There are however some studies of running which will be further dealt with in the discussion of the results of the experiments.

Routine of Experiment and Methodics.

The work comprises walking and running on a flat-belt conveyor, a "treadmill", which is driven by electricity and can be adjusted to the desired speeds with great accuracy. During the experiment the time of 20 revolutions of the belt is frequently ascertained to determine the exact speed. Moreover the number of steps per minute is counted several times in the course of each experiment. The respiratory exchange is determined by means of the Douglas-bag method. For practical reasons the experiments could not be performed in the morning on an empty stomach; they were performed in the afternoon about 3 hours after a light lunch.

The experiment is performed as follows: — When the conveyor has been adjusted approximately at the desired speed the subject begins working, the speed is controlled and is soon adjusted at the speed desired. With very few exceptions the subject was working for 10 minutes before the metabolism was determined in order to make sure that he was in a "steady state". That 10 minutes is a sufficiently long introductory period will appear from experiments by M. NIELSEN and O. HANSEN among others, showing that in a work on the bicycle ergometer of 1,620 mkg./min. with an oxygen absorption of 3.5 litres per minute the oxygen absorption will already after 5 minutes' work be constant for the rest of the experimental period, e. g. 20 minutes. As a rule 3 determinations are made every day on each subject at varying speeds with a short period of rest between the single experiments.

The subjects were the following men: —

	Weight, kilos	Height, cm.	Length of leg (from spina il. ant. sup.)	Length of tibia (from med. artic. line of knee)	Age
H. S.	67.7	174.7	99.0	47.2	31
E. A.	64.0	173.3	96.5	45.6	26
S. A.	61.0	168.9	94.0	43.1	25
I. B.	66.1	170.0	96.3	45.6	31
V. B.	71.5	181.8	105.6	49.0	22
E. O.	70.0	174.0	—	—	22
O. M.	62.5	165.0	91.0	43.9	23
O. S.	86.1	189.2	107.4	52.2	23
O. B.	75.0	184.0	102.7	47.7	36
E. K.	84.1	183.5	104.6	48.5	26
V. I.	63.4	172.1	94.5	44.2	29

H. S. has been for several years Danish champion in 5 and 10 kilometres' running, E. A., S. A., I. B. and V. B. are good medium and long distance runners; E. O., O. M., O. S., E. K. and O. B. are well-trained athletes but not competition runners. O. B., however, has not been in rational athletic training for the past 7 years, and V. I. is a Danish walking champion of several years' standing over distances of 10—50 kilometres.

Results of Experiments.

Metabolism. The oxygen absorption lies between 906 and 4450 cc. per minute and increases practically in proportion to the speed of walking or running, so long as this does not exceed what the subject can accomplish fairly easily. If this limit is exceeded the increase in oxygen absorption becomes comparatively larger, as will appear from the experiments with the rather untrained subject O. B. (Fig. 1).

The oxygen consumption in walking and running at the same speed is generally not the same. It is a well-known fact that it is more economical to walk at slow rates of speed and run at greater speeds. This is illustrated by fig. 1. It will be seen that when the speed exceeds 8 km./h. running becomes more economical. Fig. 2 shows the curves for the oxygen absorption in walking and running respectively in the best walker V. I. and the best runner H. S.; here the intersection of the curves is actually as high as 10 km./h. This is doubtless due to the fact that in this experiment it has been possible to carry through the walking with especially good economy, the walker in question having many years' training in walking-races. As the walker, however, weighed 4.3 kg. less than the runner it would not be correct to

make a direct comparison between their oxygen absorptions, and if we compare the oxygen absorption per kg. body weight the intersections of the curves will be about 9.3 km./h.

As already referred to, it is of importance, when estimating the influence of the different forms of athletics on the organism, to know the increases in metabolism they involve, for by this means we have a good measure for the strain they put on

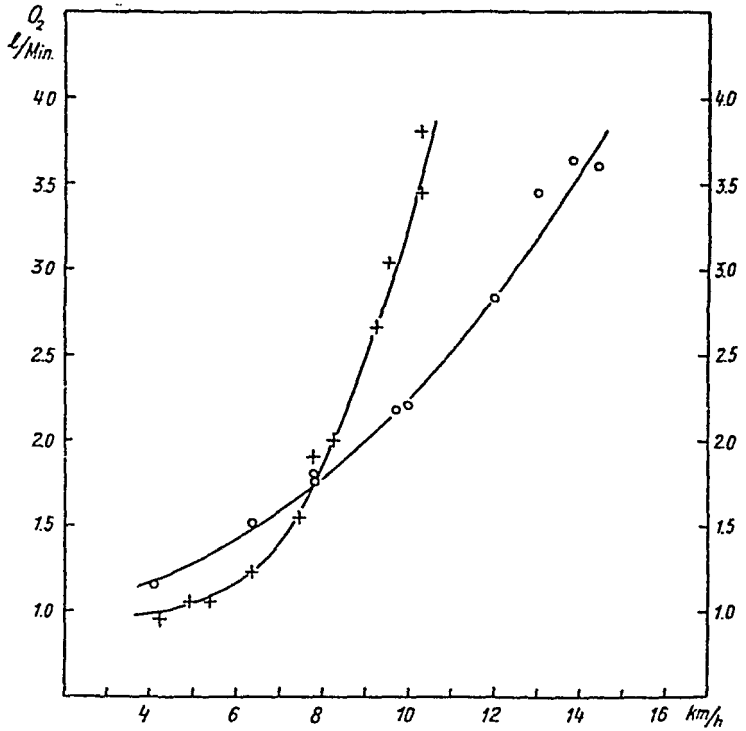

Fig. 1.

O. B. + walking,
○ running.

respiration, circulation, heat regulation, and energy production. Presuming that the values of oxygen absorption found in this series of experiments correspond fairly well to those found in walking and running on the ground, it is possible to compute the energy production in some of the long walking and running distances in common use in competitions. The results obtained on a treadmill will, of course, be minimum values, as the subjects remain in the same place and have no air-resistance to overcome, as they have in outdoor running. Anyone who has ridden a bicycle or run out of doors knows that this air-resistance plays

an important rôle. According to A. V. HILL the air-resistance in kg. can be computed from the following formula:

$$0.056 \times v^2 \times A$$

where v is the sum of (in a following wind the difference between) the runner's speed and that of the wind in m./sec, and A is the anterior surface of the body in m^2 (in these computations estimated at $^1/_2$ m^2). If the number of kilos computed in this manner

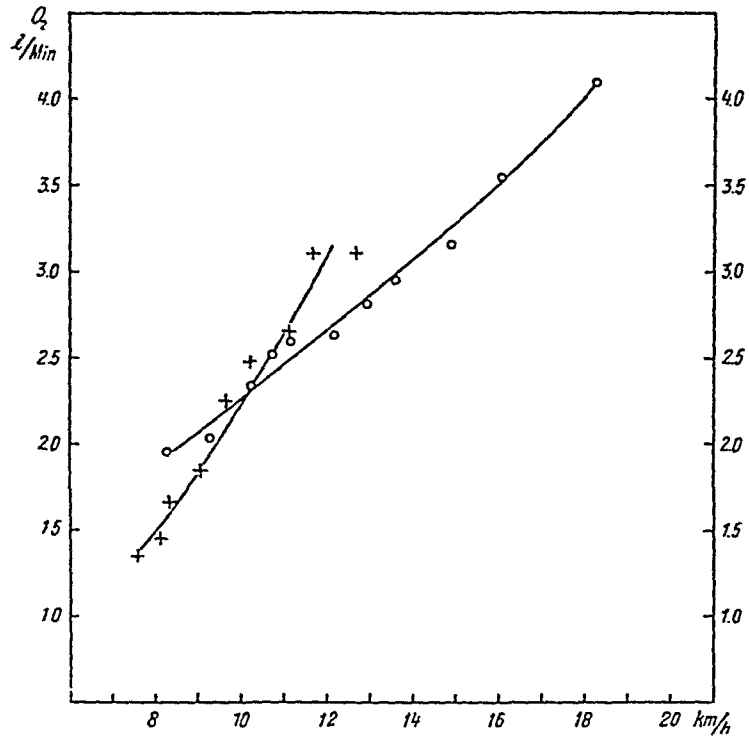

Fig. 2.
+ V. I. walking,
○ H. S. running.

is multiplied by the speed of running per minute we get the number of mkg/min. by which the air-resistance has increased the work of running. If we assume the same efficiency as is found in work on the bicycle ergometer, e. g. about 25 per cent., we can compute the increase in metabolism produced by the air-resistance at the different speeds and then again compute how much this would increase the oxygen absorption.

A few instances will be given showing what oxygen absorption the best walker and the best runner have when walking and running at varying speeds, according to the oxygen absorp-

tion measured in walking and running on the treadmill. The figure in parenthesis indicates the amount of oxygen by which the air-resistance at these speeds would have increased the metabolism according to the above computation, if the subjects had been running on the sports ground.

In a walking race over 20 km., which the subject V. I. can carry through at an average speed of about 12,5 km./h., an oxygen consumption of about 3.3 litre/min. is required (+ 0.140). Walking 50 kilometres the same man has an average speed of a little more than 11 km/.h., for which about 2.6 litres of oxygen per minute (+ 0.090) are required.

Corresponding computations for running show that a man who runs a Marathon race in Danish record-time, e. g. $2^2/_3$ hours, the distance being about 42 km., has an average speed of just under 16 km./h., which in the case of the runner H. S. requires an oxygen absorption of 3.5 litres per minute (+ 0.280). In a race of for instance 10 kilometres, which the subject can run at an average speed of 19,5 km./h., the oxygen absorption must be about 4.5 litres/min. (+ 0.500). In this connection it should be mentioned that ROBINSON and his collaborators have measured the oxygen absorption of some world-record runners running on a treadmill. The best of these runners had an oxygen absorption of 5.35 litres/min. (+ 0.730) at an average speed of 21.6 km./h. This is almost the speed at which this athlete runs a 2 miles' race in competitions. It will appear from these instances that the medium distance races require an enormous oxygen absorption and thus a corresponding function of organs of respiration and circulation. If the oxygen absorption by which the air-resistance — according to these computations — should have increased the work of running out of doors are added to the oxygen absorptions found in the work on the treadmill, we find that a 10 kilometres' race in the best time would require an oxygen absorption of about 5 litres/min. and a 2 miles' race 6.08 litres/min. As an oxygen absorption of more than 6 litres/min. is hardly possible, this means that in these races an increasing oxygen debt must be an essential factor.

In order to compare the rates of metabolism of the individual subjects at the varying speeds of running and walking we have computed the consumption of calories per kg. body weight and per kilometre at the different speeds. As will appear from Table 1, the consumption of calories in running at the speeds examined

here (8.13—18.60 km./h.) lies between 0.86 and 1.15 but, independent of the average speed, by far most values lie about 1 calorie per kilogram and kilometre at the speeds examined here, so that the energy requirement is the same in running one kilometre at the rate of 8 and at the rate of 18 km./h. But this will doubtless only hold good in the case of well-trained athletes like those employed in our experiments; none of our subjects were urged to such an extent as to make them work at their maximum capacity. At higher speeds the metabolism must be expected to increase at a still higher rate, as it has been shown, e. g. by experiments on the ergometer bicycle, that the efficiency decreases when the subject has to work near the limit of his capacity.

If the results of the experiments communicated here are compared with MARGARIA's results it will be seen that on an average his subjects have a larger energy requirement than ours, especially at the lower speeds, which is presumably due to the fact that his subjects were not so well-trained. MARGARIA has only few determinations of metabolism in running on a horizontal surface and only a single one at a speed of more than 12.5 km./h., but within the speeds examined he, too, finds approximately the same consumption of calories per kilometre run. This is at variance with the results of LILJESTRAND and STENSTRÖM, who find a far smaller requirement of calories at the high speeds. This is presumably due to the fact that these investigators have used too short an introductory period (0.4—2.2 minutes, in most cases about 1 minute) so that at the higher rates of work the subjects have not managed to get into a "steady state" before the determination of metabolism was made. Lastly, OGASAWARA's investigations must also be mentioned. He determines the metabolism of a subject running out of doors by means of the Douglas-bag method and computes the total oxygen consumption during the run by determining it before, during, and after the run and adding the oxygen-debt to the oxygen consumption found during the run itself. He finds a far greater energy transformation than we do; already at an average speed of 12 km./h. the subject, who weighs 63 kg., has an oxygen consumption of 3,858 cc. per minute, whilst in our subjects we find an oxygen consumption of about 2,700 cc. at this speed. Even if OGASAWARA's subject runs on the ground and not on a treadmill like MARGARIA's and our subjects, the difference in oxygen absorption is still

strikingly large; more-over, an oxygen absorption of nearly 4 litres/min. in running at so low speed as 12 km./h. can hardly be of general validity, not even when the subject runs on the ground, as the additional oxygen consumption, due to the air-resistance at this speed, only amounts to a little more than 100 cc. of oxygen. OGASAWARA's high figures can therefore only be explained like this: Either his subject has been untrained, or he must have added more than the true oxygen-debt, when adding the increase of metabolism after the run.

As referred to in the introduction, one object of this series of experiments was to ascertain whether the best runners have a more economical technique than the less good ones, and to find out whether the better results of the former could partly be explained in this way. As will appear from Table 1., the consumption of calories per kilometre and kilogram is practically the same both for the different speeds and the different subjects. Judged by these determinations it cannot be a difference in working economy that makes one competition runner achieve more than another, or else the difference is so slight as to be below the limit of error of the experimental technique employed.

Table 1.

The table gives the calories consumed per hour, kilometre and kg. body-weight for the different rates, these are stated in whole kilometres, omitting fractions in order to simplify the table.

Running.

km./hour

	8	9	10	11	12	13	14	15	16	17	18
H. S.		1.05		1.02		0.96		0.98		0.99	1.02
I. B.	1.06	1.07		1.02		0.96	0.94	0.96	0.98	0.98	1.00
E. A.		0.95	0.95	0.99	0.92	0.87	0.94	0.86	0.94		
S. A.			1.05	0.98		1.02			1.00	1.06	1.01
V. B.				0.96	1.06		0.96		1.00		0.98
E. O.			1.01	0.96		0.96	0.94				0.86
O. S.	1.08		1.01	1.02	1.05		1.00	0.93			
E. K.		0.98	0.96		1.00	1.08	1.01	1.03	0.99		
O. M.	1.14	1.15	1.11	1.08		1.04	1.07				
O. B.	0.88		0.88		0.93	1.08	1.00				

Walking.

km./hour

	4	5	6	7	8	9	10	11	12	13
O. B.	0.83	0.78	0.75	0.82	0.95	1.12	1.36	1.49		
V. I.					0.91	0.95	1.07	1.11	1.24	1.14

Regarding conditions in walking, we have examined 2 persons only, viz. V. I., who is a very competent walker, and O. B., who has never gone in for walking races as a sport. (See Fig. 1 and 2. and Table 1.) At an average speed of little more than 8 km./h. the trained walker's metabolism is less than that of the untrained one and the difference increases with increasing rates of speed. This very considerable difference is doubtless due to the fact that the comparison took place between a highly trained walker and a person who had never practised the difficult technique of this sport.

Pulmonary ventilation and depth of respiration. The pulmonary ventilation increases in a fairly direct ratio to the increase in oxygen absorption. In several of the subjects the well-known phenomenon is, however, observed: That when the oxygen absorption becomes very large the pulmonary ventilation increases more than in proportion to the oxygen absorption; this is seen especially in the two subjects who had had the least amount of training, Table 2., The largest ventilation per litre oxygen absorbed was seen in the subject O. B., who had had least special training and who has not had any rational training in any sport for the past 7 years, while the best of the runners, H. S., had the least ventilation per litre oxygen absorbed; this is especially pronounced when the oxygen absorptions are large. It will moreover be seen that this subject has practically the same ventilation per litre of oxygen at all rates of work, which doubtless indicates that his mechanism of respiration is very well-adjusted to the given conditions, owing to his good state of training, which is one of the reasons why no considerable accumulation of lactic acid takes place in the blood and interferes with the regulation of the respiration. The large difference between the ventilation per litre of oxygen in trained and untrained subjects is clearly seen from a comparison between the walker V. I. and O. B. in the walking experiments. The fact that trained individuals do not require such a large ventilation at a given oxygen absorption as untrained ones is doubtless of no slight importance to their capacity for the more intense achievements, where the ventilation may become the limiting factor for the performance.

In most of the subjects the depth of respiration is nearly the same at all rates of work; in a few subjects it increases a little with increasing ventilation. In H. S. it increases from 1.91 litres to 2.40 when the rate of speed is increased from 9.3 to 17.75 km./h. In I. B. it remains almost constant at 2.6 litres at all

speeds. In O. B. it increases evenly from 1.48 to 3.10 when the rate of speed is increased from 4.0 to 14.38 km./h. It will appear from this that already at a moderate rate of work most of the subjects adjust their respiration to a certain depth, whilst one of the subjects, O. B., has an evenly increasing depth of respiration with increasing ventilation. This individual difference, which does not seem to depend on the state of training, has been previously demonstrated by E. HOHWÜ CHRISTENSEN (1932) in work on the bicycle ergometer.

Table 2.

Pulmonary ventilation (37° and barometric pressure) per litre oxygen-intake.

Running.

	km./hour										
	8	9	10	11	12	13	14	15	16	17	18
H. S.		19.8		18.7		19.8		19.1		19.6	18.2
I. B.	18.2	17.4		18.4		18.2	19.0	21.5	19.4	22.4	21.3
E. A.		18.4	21.1	18.8	18.3	19.0	17.2	18.8	17.0		
S. A.			19.3	20.9		20.8			22.0	22.4	21.6
V. B.				20.3	19.1		18.5		19.7		21.6
E. O.			20.2	21.3		19.1	19.1				21.9
O. S.	17.8		19.4	20.1	19.4		20.9	22.4			
E. K.		17.9	17.9		17.8	21.8	19.4	20.3	24.6		
O. M.	19.9	18.4	18.5	20.1		21.0	22.7				
O. B.	22.4		22.9		23.7	25.2	25.9				

Walking.

	km./hour									
	4	5	6	7	8	9	10	11	12	13
O. B.	21.9	22.0	23.1	23.3	22.9	24.1	26.8	26.7		
V. I.					20.9	20.0	18.5	19.4	20.9	22.0

The slight depth of respiration (on an average 1.6 litres at all speeds) and the high frequency of respiration (increasing evenly from 23 to 44 per minute) of the walker V. I. are remarkable features, which are due, presumably, to the fact that during walking races the respiration is less free than during running, because at a quick walk the abdominal muscles are constantly rather contracted, thus interfering with the respiration. In this connection it should, however, be noted that in the subject O. B. the depth of respiration is not less when he walks than when he runs at the same oxygen absorption, which may be due to a difference in technique of walking in the two subjects; there-

fore an examination of several walkers is required before anything definite can be said about this point.

The length of steps at the different speeds of running and walking. To increase our speed when walking or running we must either take more steps per minute, or take longer steps, or we may increase both the length and the frequency of the steps. One would expect the third method to be the one actually resorted to, but it appears that with increasing speed the frequency of the steps is little increased whilst, on the other hand, the length of the steps is greatly augmented. As an instance may be mentioned that at an average speed of 9.3 km./h. the frequency of steps in H. S. is 160 per minute and at a speed of 17.75 km./h. it is 176, the corresponding lengths of steps being 97 and 168 cm. respectively. In the case of A. S. the corresponding figures are 160 and 182 and 102 and 168. The other subjects showed similar results. Running correctly at the speeds examined here (8—18 km./h.) the runner will thus preferably increase the speed by augmenting the length of his steps.

The explanation of this tendency to keep a fairly constant frequency of steps, irrespective of the speed, may perhaps be sought in the fact that within the speeds examined here the movements of the legs in certain phases may take place as pendulum movements and consequently be of a definite rhythm; if the latter has to be retarded or urged on it will demand additional muscular activity.

As far as walking races are concerned we counted the frequency of steps for one subject only, V. I. It appeared that the frequency of steps increased evenly from 158 per minute at a walking speed of 7.50 km./h. to 188 at a speed of 12.78 km./h., and the length of steps from 79 to 113 cm. Thus in walking there seems to be a greater tendency to increase the frequency than to increase the length of steps. This tallies well with the theories advanced above in connection with running: It is a well-known fact that when we walk the legs do not move like pendulums, but have to be moved forwards by active muscular energy alone. At a quick walk this forward movement of the legs becomes very powerful, because many of the muscles of the trunk and the arms have to function in order to swing the legs forwards. This fact explains why a quick walk is more exerting and requires a greater energy transformation than running at the same speed.

Summary.

The object of the present paper is to elucidate some of the factors determining the difference of efficiency between the best runners and other well-trained individuals who have not gone in for running with a special view to competition. A single prominent walker has also been included in the series of experiments. Determinations of metabolism, frequency and volume of respiration and number and length of steps were made in subjects who were running or walking on a flat-belt conveyor driven by a motor.

The oxygen absorption found varied from 906 to 4,450 cc. per minute and increased practically in a direct ratio to the rate of running and walking, as long as this was what the subject could manage fairly easily. If this limit was exceeded, the oxygen absorption increased in a comparatively steeper curve. At a quick walk a larger oxygen absorption was found than at a run at the same speed.

The production of calories per kg. body weight and km. was practically the same at all speeds of running between 8 and 18 km./h., whilst it was increased in walking at the higher speeds. There was no certain difference in the consumption of calories per kg. body weight and km. in the different trained runners, while it was a little higher in untrained subjects; this difference was even larger in walking.

The pulmonary ventilation increased in proportion to the oxygen absorption; in the least well-trained subjects there was a disproportionately large increase in the ventilation at the largest oxygen absorptions, and the ventilation per litre of oxygen absorbed was highest in the least trained subject.

In most of the subjects the depth of respiration was nearly the same at all rates of ventilation. In a single subject, however, the depth of respiration increased evenly with the rate of ventilation. In walking races a comparatively slight depth of respiration was found.

The frequency of steps is only slightly increased with increasing speed in running, but the length of the steps is greatly increased; but in walking races there seems to be a greater tendency to increase the frequency of steps at increasing speeds.

Literature.

CHRISTENSEN, E. HOHWÜ, Arb.physiol. 1932. *5*, 463.
HILL, A. V., Proc. Roy. Soc. B 1928. *102*, 380.
LILJESTRAND, G. and N. STENSTRÖM, Skand. Arch. Physiol. 1920. *39*, 167.
MARGARIA R., Reale Acc. dei Lincei 1938. Ser. 6. *7*.
NIELSEN, M. and O. HANSEN, Skand. Arch. Physiol. 1937. *76*, 37.
OGASAWARA M., J. Physiol. 1934. *81*, 255.
ROBINSON S., H. T. EDWARDS and D. B. DILL, Science 1937. *85*, 409.

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On the Affinity of Pig Pancreas Lipase for some Lower Triglycerides in Homogeneous Solution.

By

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Only a few investigations on the affinity of pig pancreas lipase for lower triglycerides in homogeneous solution have been reported. SOBOTKA and GLICK (1934) have determined the affinity of pig pancreas lipase for tributyrin and found Michaelis constant $K_s = 6.00 \times 10^{-4}$. No activators were used. WEINSTEIN and WYNNE (1935—36) have examined the hydrolysis of tripropionin by pancreas lipase in the presence of egg albumin, gum acacia and calcium chloride. Velocities calculated from the Michaelis equation did not agree satisfactorily with the observed velocities. KRÄHLING and WEBER (1938) have determined K_s for pig pancreas lipase towards triacetin in solution without and with sodium oleat and calcium oleat. In all cases they found the same $K_s = 0.25$.

It appears from the following paper that it has not been possible to verify the above mentioned K_s values. With five of the lowest triglycerides no deviation from proportionality between substrate concentration and lipase activity was found in the range where the triglyceride solutions were homogeneous with certainty. Calcium chloride, bile salts and egg albumin do not change the relationship between substrate concentration and enzyme activity. The relative velocities of the hydrolysis of the triglycerides in homogeneous solution are embodied in the present report.

Experimental.

Substrates. Triacetin, tripropionin, tributyrin, trivalerin and tricaproin were used for the experiments. The solubities of these triglycerides in water at 30°C were determined by the method of SOBOTKA and KAHN (1931). To known amounts of water a little sudan was added. Then triglyceride was added dropwise from a graduated 0.1 cm³ pipette. When the solution is saturated with triglyceride the sudan will be taken up by the fat particles, and the presence of transparent globes, demonstrated by aid of a magnifying glass, is taken as a criterion of saturation. The results are given in Table I which also contains the molecular weight determined by saponification (M_{exp}) and d_4^{20}.

Table I.

Solubility of Triglycerides in Water at 30° C.

Triglyceride	M_{exp}	d_4^{20}	g per 1 solution	Molecules per 1 solution
Triacetin	223	1.154	73.2	0.328
Tripropionin	260	1.080	5.34	0.0205
Tributyrin	302	1.030	0.20	6.6×10^{-4}
Trivalerin	365	1.011	0.038	1.04 — —
Tricaproin	407	0.981	0.0045	0.11 — —

Enzyme. Pig pancreas lipase has been prepared according to the method of WILLSTÄTTER and WALDSCHMIDT-LEITZ (1923). According to the different activity of lipase towards triglycerides the glycerol extract was used undiluted or in suitable dilutions with glycerol. The enzyme solutions were kept in ice or in the ice-box. In order to work with constant enzyme activity the K_s determinations for each triglyceride were carried out in the course of 1 or 2 days.

Determination of the enzymatic hydrolysis. The measurement of the lipase activity was carried out by a modification of the method of KNAFFL-LENZ (1923), not using indicator but measuring p_H electrometrically by means of a protected glass electrode and a Radiometer potentiometer. The experiments took place in a water thermostat at 30°C $\pm$ 0.2° in a small isolated room kept at the same temperature. The glass electrode and the reference electrode were inside this room, the potentiometer outside at ordinary room temperature. The p_H in the reaction mixture was kept at about 7.2 to 7.5 by adding dropwise 0.01 to 0.04 n NaOH to neutralize the acid liberated as fast as it was formed during the enzymatic hydrolysis. The number of drops (1 cm³ = about 40 drops) added during the first 10 min. of the reaction give a relative measure of the initial velocity. This value is computed graphically from the curve showing the relation between added drops and time. In the course of 8 to 20 min. it is possible to get 6 to 8 points on the curve, which is generally linear in the beginning in agreement with the degree

of hydrolysis not exceeding 10 per cent. In the case of trivalerin and tricaproin at the lowest substrate concentrations it was only possible to get 2 to 3 points on the curve. According to the solubility in water the amount of substrate solution varied from 20 cm^3 (triacetin) to 500 cm^3 (trivalerin and tricaproin). In most of the experiments small amounts of Michaelis veronal buffer (0.4 to 1 cm^3) with $p_H = 7.7$ were added to the mixture to avoid too great p_H displacements. The veronal buffer does not influence the reaction velocity. The reaction was started by dropping small glass vessels containing 0.1 to 2.0 g of enzyme into the substrate solution which beforehand had been adjusted to the proper p_H.

The following experiment demonstrates the technique in details. The solution contained 0.8921 g triacetin + 0.4 cm^3 veronal buffer + 17.7 cm^3 water + 0.9999 g enzyme (1 : 10). — Immediately after the addition of enzyme p_H was measured. By addition of 0.04 n NaOH dropwise the p_H was brought above this value, see Table II. By interpolation the times corresponding to the initial p_H were determined. Number of drops were plotted as ordinate with time as abscissa. The inclination of the straight line gives the initial velocity = 25.7 drops of 0.04 n NaOH per 10 min.

Table II.

Hydrolysis of Triacetin.

Time in min.	p_H	Number of drops added	Time corresponding to $p_H = 7.40$
0 (start)			
1.00	7.40	0	1.00
1.02	7.42	3	2.10
2.07	7.335	»	
3.07	7.41	7	3.77
4.58	7.32	»	
5.33	7.405	11	5.38
6.00	7.345	»	
7.58	7.442	18	8.04
8.75	7.335	»	
9.67	7.395	22	9.59
10.08	7.370	»	

Result: 25.7 drops 1/25 n NaOH per 10'.

In some cases single determinations with various amounts of enzyme towards the same substrate concentration have been carried out, in other cases double or triple determinations with one amount of enzyme towards the same substrate concentration, after having shown in preliminary experiments that there is no deviation from proportionality between enzyme concentration and velocity in the enzyme-substrate concentration range examined. In the case of triacetin, tripropionin and tributyrin the velocity measured has to be corrected for spontaneous hydrolysis, which is not negligible. With trivalerin and tricaproin no spontaneous hydrolysis could be demonstrated.

The following experiments on tripropionin give a typical example which demonstrates the accuracy of the method employed. The reaction mixture consisted of tripropionin in different concentrations, 1 cm³ of veronal buffer, varying amounts of enzyme solution and water, 50 cm³ in all. p_H was adjusted by addition of 0.02 n NaOH. v is given as 2 × the number of drops per 10 min. The degree of hydrolysis did not exceed 5 per cent, in most cases it was less than 3 per cent. The results are to be found in Table III, showing close proportionality between velocity and substrate concentration. The coefficient in the last column (Table III) has an average of 5.00 with $\sigma = 0.46$, i.e. V = 9.2 per cent. For a double determination V = 6.5 per cent.

Table III.

Hydrolysis of Tripropionin by Pig Pancreas Lipase.

Tripropionin m × 10³	Enzyme in g	v_{uncorr}	$\frac{v_{corr}}{\text{g enz.} \times m \times 10^3}$
1.60	1.0001	9.5	5.44
»	2.0001	16.3	4.85
3.00	1.0000	14.2	4.24
»	1.5001	24.5	5.23
»	2.0001	30.2	4.79
6.00	0.4999	16.5	4.51
»	1.0000	32.4	4.90
»	1.5000	45.4	4.71
9.00	0.2500	15.4	4.87
»	0.5000	31.2	5.94
»	1.0000	51.6	5.24
15.01	0.2501	25.3	4.77
15.00	0.5000	48.9	5.52

Results.

Table IV contains the results of experiments with five different triglycerides in homogeneous solution. The velocities given are average values of double determinations. The reaction mixtures had the following composition:

Triacetin: Triacetin + 0.4 cm³ veronal buffer + enzyme solution + water, 20 cm³ in all. Titration with 0.04 n NaOH.

Tripropionin: Tripropionin + 1 cm³ veronal buffer + enzyme solution + water, 50 cm³ in all. Titration with 0.02 n NaOH.

Tributyrin: Tributyrin + 1 cm³ veronal buffer + enzyme solution + water, 100 cm³ in all. Titration with 0.02 n NaOH.

Trivalerin and Tricaproin: Triglyceride + enzyme solution + water, 500 cm³ in all. Titration with 0.01 n NaOH.

Table IV.

The Effect of Varying Substrate Concentrations on Reaction Velocity.

Triglyceride	m	Enzyme in g	v_{corr} / g	v_{corr} / g × m	V in per cent
Triacetin	2.00×10^{-2}	1.000	4.33	2.17×10^{2}	
	4.00 — —	1.000	8.42	2.11 — —	
	10.00 — —	1.000	22.00	2.20 — —	
	16.00 — —	1.000	33.74	2.11 — —	
	20.00 — —	1.000	49.30	2.46 — —	
			average	2.21 — —	6.6
Tripropionin	1.99×10^{-3}	1.200	10.85	5.35×10^{3}	
	3.99 — —	1.200	20.65	5.17 — —	
	5.98 — —	1.200	35.8	5.96 — —	
	8.57 — —	1.200	48.3	5.63 — —	
	11.50 — —	1.000	66.3	5.76 — —	
	13.00 — —	0.800	70.4	5.42 — —	
	17.00 — —	0.800	97.5	5.74 — —	
			average	5.57 — —	4.9
Tributyrin	0.90×10^{-4}	0.500	25.35	28.2×10^{4}	
	1.80 — —	0.500	58.5	32.5 — —	
	3.60 — —	0.250	92.8	25.8 — —	
	4.80 — —	0.250	136.3	28.4 — —	
	5.92 — —	0.250	172.0	29.1 — —	
			average	28.8 — —	10.1
Trivalerin	1.04×10^{-5}	0.600	46.7	44.8×10^{5}	
	2.08 — —	0.600	96.0	46.1 — —	
	4.16 — —	0.600	179.5	43.1 — —	
	6.24 — —	0.300	320.0	51.2 — —	
			average	46.3 — —	7.9
Tricaproin	0.365×10^{-5}	2.000	10.33	28.3×10^{5}	
	0.730 — —	2.000	18.4	25.2 — —	
	1.09 — —	2.000	29.7	27.2 — —	
			average	26.9 — —	5.9

m = molarity of substrate. v = 2 × number of drops NaOH in 10 minutes.

It appears from Table IV that $v_{corr} = K \times g \times m$, i.e. the corrected velocity per g of enzyme is proportional to substrate concentration for the triglycerides examined in homogeneous solution. In the experiments described in Table III a variation coefficient, V, of 6.5 per cent was found for double determinations. V, given in Table IV agrees fairly well with this value. With a finite K_s value one would expect decreasing of the ratio $v_{corr}/g \times m$. In none of the experiments this ratio showed a trend to decrease. According to Michaelis' theory this means that K_s is ∞ or very

large compared with the substrate concentration measured. The affinity, which is the reciprocal of K_s, is thus very small.

It ought to be mentioned that the reaction velocity increased abruptly far beyond proportionality when the amount of substrate per unit of volume exceeded the saturation limit, but in heterogeneous system without activators the reaction velocities could not be reproduced with the accuracy wanted. We hope to return to this question in a later publication.

WILLSTÄTTER, WALDSCHMIDT-LEITZ and MEMMEN (1923) and WILLSTÄTTER and MEMMEN (1924) explain the effect of activators (protein, bile salts a. o.) on lipases by the formation of complex adsorbates of enzyme and substrate. Some experiments were undertaken to examine the possible influence of some of the activators on the K_s value. As bile salt sodium taurocholate was used in one experiment and in the rest — owing to the impossibility of getting more sodium taurocholate — dried ox bile in glycerol, prepared according to the method of BALLS, MATLACK and TUCKER (1937—38). In preliminary experiments the optimum concentrations of the activators were determined. The substrate in these experiments was 50 cm³ of 6×10^{-3} m tripropionin, containing varying amounts of activators. No acceleration could be proved with certainty for egg albumin. The optimum concentration for sodium cholate was 640 mg per cent and for ox bile in glycerol 10 per cent by volume, the maximum increase being about 50 per cent. By addition of calcium chloride the velocity rose rapidly reaching a level at about 80 mg per cent calcium chloride, the maximum increase being about 300 per cent. On the basis of these experiments the following concentrations were chosen: 640 mg per cent sodium taurocholate, 10 per cent by volume of ox bile in glycerol, 200 mg per cent calcium chloride and 200 mg per cent egg albumin.

Table V summarizes the results of our experiments with addition of calcium chloride, bile salts and egg albumin. In these experiments, too, proportionality was found between substrate concentration and enzyme activity. The very low affinity found in the systems investigated does not make it possible to decide with certainty whether the activators influence K_s.

KRÄHLING and WEBER (1938) have not been able to demonstrate any influence of activators on the affinity of pig pancreas lipase for triacetin. These authors find for triacetin without and with activators a finite K_s value equal to 0.25. However, when the

Table V.

Effect of Varying Substrate Concentration on Reaction Velocity in the Presence of Activators.

Triglyceride	m	Enzyme in g	$\frac{v_{corr}}{g}$	$\frac{v_{corr}}{g \times m}$	V in per cent
Triacetin	2.00×10^{-2}	0.500	26.7	13.35×10^2	
	5.00 — —	0.500	64.3	12.86 — —	
	10.00 — —	0.500	129.0	12.90 — —	
	16.00 — —	0.250	205.6	12.85 — —	
	26.00 — —	0.250	331.6	12.75 — —	
			average	12.95 — —	1.78
Tripropionin (I)..	1.99×10^{-3}	0.500	41.1	20.65×10^3	
	4.98 — —	0.500	116.1	23.3 — —	
	13.00 — —	0.250	239.3	18.4 — —	
	17.00 — —	0.250	404.0	23.8 — —	
			average	21.54 — —	11.6
Tripropionin (II).	2.17×10^{-3}	0.400	22.5	10.36×10^3	
	4.34 — —	0.500	53.6	12.35 — —	
	8.14 — —	0.500	85.0	10.45 — —	
	13.04 — —	0.400	138.5	10.62 — —	
	15.01 — —	0.300	179.5	11.95 — —	
	17.00 — —	0.250	195.0	11.45 — —	
			average	11.20 — —	7.5
Tributyrin	0.79×10^{-4}	0.500	33.85	42.6×10^4	
	1.59 — —	0.500	66.5	41.8 — —	
	3.18 — —	0.250	160.5	50.4 — —	
	4.52 — —	0.250	214.4	47.4 — —	
	5.42 — —	0.250	266.0	49.9 — —	
			average	46.0 — —	8.4

m = molarity of substrate. v = 2 × number of drops NaOH in 10 minutes. The triacetin solutions contained: $CaCl_2$, egg albumin and ox bile in glycerol. Tripropionin I: $CaCl_2$, egg albumin and sodium tauro-cholate. Tripropionin II: $CaCl_2$ and ox bile in glycerol. Tributyrin: $CaCl_2$ and ox bile in glycerol.

substrate concentrations and velocities are calculated from their graphic representation and an attempt is made to compute K_s graphically from the straight line $\frac{m}{v} = \frac{m}{v_\infty} + \frac{K_s}{v_\infty}$ it appears that the points fall badly on a straight line, K_s thus being determined inaccurately. WEINSTEIN and WYNNE (1935—36) have tried to determine K_s for tripropionin by measuring the velocity at 10 different concentrations. When their results did not agree satisfactorily with the Michaelis theory, this is perhaps due to the fact that they have worked in heterogeneous systems. According to our investigations the solubility of tripropionin in water is 0.0205

m, and WEINSTEIN and WYNNE state to have measured in the concentration range 0.016 m to 0.16 m. As previously mentioned SOBOTKA and GLICK (1934) have found a finite K_s for tributyrin and pancreas lipase. According to their data this value is determined with great accuracy. We have have not been able to verify these authors' findings neither with the enzyme preparations mentioned in this paper nor with other enzyme preparations, and we do not understand how SOBOTKA and GLICK can extend their homogeneous range to 22×10^{-4} m tributyrin.

As the experiments mentioned in Table IV and V were carried out with different samples of enzyme solutions the $v_{corr}/g \times m$ values are not comparable. For the purpose of comparison the initial velocities for the different triglycerides in homogeneous solution were determined in experiments which were carried out in one day with the same enzyme preparation in varying amounts. No activators were used. Table VI shows the relative initial velocities for the same amount of enzyme and substrate concentration. The figures are related to an arbitrary value of 100 for tributyrin. It is seen that tributyrin is split with maximum initial velocity in agreement with the results previously published by the authors (SCHØNHEYDER and VOLQVARTZ, 1944). When we have not found the identical relative velocities it is probably because the system earlier investigated contained activators and only triacetin and tripropionin were present in homogeneous solution.

Table VI.

Relative Initial Velocity of Hydrolysis of Triglycerides by Pig Pancreas Lipase in Homogeneous Solution.

Triglyceride	$v_{rel.}$
Triacetin	0.062
Tripropionin	3.25
Tributyrin	100
Trivalerin	10.7
Tricaproin	27.3

Summary.

The affinity of pancreas lipase for triacetin, tripropionin, tributyrin, trivalerin and tricaproin has been investigated in *homogeneous* solution without activators and in the presence of bile salts, calcium chloride and egg albumin.

1. The solubilities in water at 30°C were determined for the triglycerides examined.
2. The initial velocity of hydrolysis varied directly as the enzyme concentration.
3. The initial velocities were directly proportional to substrate concentration in the systems without and with activators. The affinity of pancreas lipase for the triglycerides in homogeneous solution thus being extremely low.
4. The relative initial velocities for the hydrolysis of five triglycerides in homogeneous solution by pancreas lipase are given.

Professor, Dr. Hakon Lund, Chemical Institute, Aarhus, has kindly prepared some of the triglycerides used.

References.

BALLS, A., M. MATLACK and I. TUCKER, J. Biol. Chem., 1937—38, *122*, 125.
KNAFFL-LENZ, F., Arch. exp. Path. Pharmak. 1923, *97*, 242.
KRÄHLING, K., and H. H. WEBER, Biochem. Z., 1938, *298*, 227.
SCHØNHEYDER, F., and K. VOLQVARTZ, Enzymologia, in press.
SOBOTKA, H., and J. KAHN, J. Amer. Chem. Soc., 1931, *53*, 2935.
SOBOTKA, H., and D. GLICK, J. Biol. Chem., 1934, *105*, 199.
WEINSTEIN, S., and A. WYNNE, Ibidem, 1935—36, *112*, 641.
WILSTÄTTER, R., E. WALDSCHMIDT-LEITZ and F. MEMMEN, Hoppe-Seyl. Z., 1923, *125*, 93.
WILSTÄTTER, R., and E. WALDSCHMIDT-LEITZ, Ibidem, 1923, *125*, 132.
WILSTÄTTER, R., and F. MEMMEN, Ibidem, 1924, *133*, 229.

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Aus dem Cereal-Laboratorium der Upsala Ångqvarns A.B., Uppsala

Über den B_1-Vitamingehalt der Vermahlungsprodukte des Weizens und über Möglichkeiten, die B_1-vitaminreichsten Mehlfraktionen als Menschennahrung auszunutzen

Von

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Diskussion über B_1-Vitamingehalt und Ausbeute.

Ausmahlungsprozent	A. Die Passagen ausgewählt nach steigendem Aschengehalt			B. Die Passagen ausgewählt nach steigendem Gehalt an Vitamin B_1. (Kleiepassagen ausgeschlossen.)		C. Vergleichswerte nach Ziegler	
	γ % B_1	Proz. B_1 vom Ganzen	Proz. Diff.	γ % B_1	Proz. B_1 vom Ganzen	Ausmahlungsprozent	Proz. B_1 vom Ganzen
25	61,2	4,75		58,0	4,52		
			1,53				
30	67,0	6,28		61,7	5,78		
			2,91				
40	73,7	9,19		69,3	8,66	40,1	6,8
			2,91				
50	78,0	12,1		77,0	12,0		
			5,0				
60	91,2	17,1		88,3	16,6	63,5	17,4
			4,1				
65	105	21,2		101	20,5		
			9,3				
70	140	30,5		126	27,6	70,45	24,5
			11,0				
75	177	41,5		167	39,1	74	31,2
			16,0				
80	230	57,5		230	57,5	81,04	56,3
			15,1				
82,5	282	72,6		282	72,6		
			5,4				
85	294	78,0					
			8,1				
90	307	86,1				92,48	88
			7,0				
95	314	93,1					
			6,9				
100	321	100					

Tabelle 1. Der Gehalt des Weizenmehles an Vitamin B_1 bei verschiedenen Ausmahlungsprozenten.

INHALTSVERZEICHNIS.

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VORWORT.

Die Untersuchungen, auf die sich die vorliegende Arbeit stützt, wurden in dem Laboratorium und der Mühle der UPSALA ÅNGQVARNS A. B. ausgeführt. Diese Firma hat bereitwillig Personal, Laboratorium und sonstige Mittel zur Verfügung gestellt. Die Verfasser möchten der Firma und deren Chef, Herrn Direktor H. W. SÖDERMAN ihren herzlichsten Dank aussprechen.

Wir sind Herrn Prof. Med. Dr. G. BLIX für wertvolle Kritik des Manuskriptes sehr dankbar.

Sämtliche Vitaminbestimmungen sind von C.-B. L. gemacht. Mit grosser Sorgfalt haben Herr S. AHLIN und Frau M. SUNDELL die übrigen Analysen und Herr S. HÖGMAN die Backversuche ausgeführt. Die Verfasser danken diesen ihren Mitarbeitern herzlichst.

Einleitung und Zielsetzung.

Dem Gehalte der Cerealprodukte an Vitamin B_1 (Aneurin) hat man in den letzten Jahren lebhaftes Interesse zugewendet, weil diese neben den Kartoffeln die wichtigste Kohlehydrat- und B_1-Vitaminquelle des Menschen ausmachen. Besonders die in den anglo-amerikanischen Ländern angeblich hohe Frequenz leichter B_1-Hypovitaminosen hat man in Verbindung gebracht mit dem geringen Aneuringehalt der dort verzehrten Cerealprodukte gegenüber deren hohem Kalorienwert. Es besteht nämlich die Meinung, dass der Bedarf an Vitamin B_1 in erster Linie von der Anzahl zugeführter Kohlehydrat- und Eiweisskalorien abhängt, wegen der Schlüsselstellung dieses Vitamins für den Kohlehydrat- und Eiweissumsatz. Da nach unseren jetzigen Kenntnissen die Cerealien im Naturzustande einen Überschuss des fraglichen Vitamins im Verhältnis zu ihren Kohlehydrat- und Eiweisswerten enthalten, so hat sich das Interesse auf die Frage konzentriert, wie die industrielle und haushaltstechnische Bearbeitung der Cerealien eine solche Herabsetzung des B_1-Vitamingehaltes zu verursachen vermag, dass dadurch Mangelerkrankung eintreten kann.

Vielfältige Untersuchungen haben festgestellt, dass der Hauptbestandteil des Weizenkornes, das Endosperm (s. Fig. 1.), das Aneurin nur in sehr geringer Konzentration enthält. Dagegen findet sich dieses völlig dominierend in Keim und Aleuronschicht, von denen die letztgenannte unmittelbar unter der schützenden Samenhülle liegt. Pulkki und Puutula (1941) konnten den Nachweis führen, dass der Gehalt der Samenhülle an Vitamin B_1 verhältnismässig niedrig und sicher geringer ist als für das Korn im ganzen.

Bei der Vermahlung des Weizens ist man bestrebt, das Endosperm von Keim und Aussenschicht zu trennen, welche durch ihren Gehalt an u. a. Fermenten und Salzen im höchsten Grade nachteilig auf die haushaltstechnischen Eigenschaften des Mehles (Backfähigkeit u. s. w.) einwirken. Im Verlaufe der Ausmahlung teilt man das Korn

in eine grosse Zahl sogenannter Mehlpassagen. Diese werden nach ihrem Aschengehalt klassifiziert, der in gewisser Weise die morphologische Herkunft der Fraktion ausdrückt, insofern als die Samenhülle den höchsten Aschengehalt hat, hiernächst der Keim und die Aleuronschicht. Das Endosperm selbst ist aschenarm. Bei Erzeugung der handelsüblichen Mehlsorten wählt und mischt man in erster Linie diejenigen Mehlfraktionen, welche den geringsten Aschengehalt haben, und spricht dann von Mehl verschiedener prozentualer

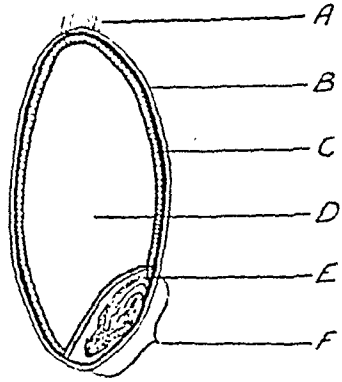

Fig. 1. Schematische Darstellung eines Weizenkornes. *A*. Bart. *B*. Samenhülle. *C*. Aleuronzellenschicht. *D*. Endosperm. *E*. Scutellum. *F*. Keim.

Ausmahlungsgrade. Unter 70% *Ausbeute* versteht man also, dass man 70% Mehl und einen Rest von 30% erhalten hat, der aus sogenanntem Nachmehl und Kleie besteht (s. S. 10).

Um eine eingehendere Kenntnis des Vitamingehaltes verschiedener Weizenmehlsorten zu gewinnen, muss man zunächst wissen, wie diese in einer modernen Grossmühle hergestellt werden. Die übliche Angabe der Vitaminziffern für Mehle von verschiedenen prozentualen Ausmahlungsgraden reicht nicht aus, sondern es bedarf einer gründlichen Analyse aller in dem Endprodukt enthaltenen „Passagen". Ausser Gehalt an Vitamin B_1 sind demnach die mühlentechnischen Variablen zu bestimmen: prozentuale Passageausbeute, Aschengehalt, Proteinmenge, Backfähigkeit und eventuell Gehalt an Fett. Des weiteren muss die Herkunft der Passage durch genaue Namenbezeichnung angegeben werden. Irgendwelche in dieser Hinsicht vollständigen Arbeiten waren nicht aufzufinden.[1] Am besten ent-

[1] Die anglo-amerikanische Literatur der Kriegsjahre auf diesem Gebiete war im grossen und ganzen nicht zugänglich.

sprechen diesen Anforderungen Untersuchungen von SCHULERUD, KANTER und ERICHSEN (1941), PULKKI und PUUTULA (1941), LITZENDORF und SCHNEIDER (1942) sowie ZIEGLER (1943). Im folgenden soll über eine Analyse aller Fertigprodukte einer modernen Weizenmühle berichtet werden. Diese Mühle ist repräsentativ für jede moderne europäische Grossmühle, weshalb die Analysenwerte als von allgemeinem Interesse angesehen werden können. Kleinmühlen teilen das Weizenkorn nicht in dieselbe grosse Anzahl Fraktionen, und die erhaltenen Werte sind deshalb nicht direkt für solche Mühlen anwendbar.

Schematische Beschreibung des Vermahlungsverlaufes in einer modernen Weizenmühle.

Das Getreide geht zunächst durch die Reinigungsabteilung, wo es durch Sieben, Lüften, Sortieren und Waschen von Fremdteilen befreit wird. Ein kräftiges Bürstverfahren entfernt lose Aussenschalenteile und Bart. In einer Anzahl Mühlen werden auch Spitz- und Schälmaschinen verwendet, mittels deren man die Weizenkeime bis zur Hälfte wegnehmen resp. einen Teil der Kornschale abschleifen kann. Im vorliegenden Falle sind diese Maschinen nicht benutzt worden.

Die Vermahlung des gereinigten Weizens geschieht zwischen liegenden Walzen in sogenannten Walzenstühlen, die in drei parallelen Serien angeordnet sind, so dass die Vermahlung in drei Etappen vor sich geht.

1. Das Getreide passiert zunächst die „Schrotungsserie". Im ersten Walzenstuhl dieser Serie wird das Korn aufgerissen. Die Absicht dabei ist, so grosse Endospermstücke wie möglich von der Schale zu lösen, ohne diese erheblich zu pulverisieren. Hinter dem Walzenstuhl befindet sich ein Sichter, welcher die Schrotungsprodukte nach deren Partikelgrösse aufteilt. Hierbei erhält man vier Fraktionen, welche als *Übergang*, *Griess*, *Dunst* und *Schrotmehl* bezeichnet werden.

a. Der „Übergang" wird nochmaliger Schrotung im Walzenstuhl 2 unterworfen, wodurch ein weiterer Satz der genannten Produkte erhalten wird. Dieses Verfahren wiederholt man fünf- bis siebenmal, wobei jedesmal die erwähnten vier Produkte anfallen. Der Übergang der sechsten und siebenten Schrotung macht die sogenannte *Kleie* aus, die aus einem Gemisch von hauptsächlich Samenschale, etwas anhängender Aleuronschicht und einer kleinen Menge Endosperm besteht.

b. Der „Griess" der verschiedenen Schrotungen wird je nach Reinheit und Partikelgrösse zu verschiedenen Gruppen vereinigt.

Diese „Griesgruppen" passieren sodann Sortiersichter zwecks weiterer Kontrolle der „Griessdimensionen", wobei etwas Abreibmehl entsteht. Sie gehen dann durch die Putzmaschinen, wo sie unter Absieben im Luftstrom von mitgenommenen Kleiepartikeln befreit werden. Das kleiehaltige Produkt, „*Koppen*", wird nochmals geschrotet.

2. In der Griessauflösungsserie wird der Griess in Partikel zerlegt, deren Grösse etwas gröber ist als die des Mehles; dieses Produkt heisst Dunst. Der Griess wird also nicht direkt in Mehl verwandelt, denn dies würde eine starke Verengung des Walzenzwischenraumes verlangen, und die dadurch entstehende Korrosion der Stärkekörner würde die Backfähigkeit des Mehles herabsetzen. Bei der Griessauflösung bildet sich indessen etwas Mehl; dieses wird abgesiebt und heisst im Protokoll Mehl der 1., 2., 3 u. s. w. *Griessauflösung.*

3. In der dritten Vermahlungsserie wird der Dunst durch 10 bis 14 in einer Serie geordnete Walzenstühle mit zwischengeschalteten Sichtern in Mehl übergeführt. Die Produkte heissen 1., 2., 3. u. s. w. *Dunstmehl.* Die fünf ersten Walzenstühle arbeiten mit einheitlichem Dunst von der Griessauflösung; von der sechsten Dunstpassage an werden jedoch gewisse dunstähnliche Fraktionen von anderen Teilen der Vermahlung zugesetzt.

Durch den Schrotungsprozess werden die Keime freigelegt, die ungefähr ebenso gross sind wie der gröbste Griess. Sie gehen deshalb mit diesem durch Sortierung und Griessputzerei bis zur ersten Griessauflösungsmaschine. Der Griess wird hier in Dunstpartikel zerlegt, während die Keime zu Scheiben gepresst werden, die man absiebt. Weitere Auswalzung und Aussortierung erfolgt in den sogenannten Übergängen.

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[1] Die Passageproben, an welchen die vorliegende Untersuchung ausgeführt worden ist, wurden in der Mühle zu einer Zeit genommen, als durch staatliche Vorschrift eine prozentual besonders hohe Mehlausbeute vorgeschrieben war. So sollten von Weizen wenigstens 70 % zu Weizenmehl und ausserdem 11 % zu Weizennachmehl ausgemahlen werden. Das letztere sollte verordnungsgemäss Roggensicht- oder Roggenschrotmehl zugesetzt werden.

Wegen der hohen Mehlentnahme war es aus mühlentechnischen Gründen notwendig, in allen Passagen das Mehl gröber zu sichten als unter normalen Umständen.

Methodik.

Chemische Analysen.

HARRIS und WANG (1941) sowie v. FELLENBERG (1942) haben festgestellt, dass gute Übereinstimmung besteht zwischen Resultaten, die einerseits durch biologische Bestimmung von Vitamin B_1, andrerseits nach JANSENS *Thiochromverfahren* gewonnen wurden, nachdem dieses durch Berücksichtigung gebundener Aneurinformen verbessert worden war, welche bei fermentativer Spaltung in Freiheit gesetzt werden. Zum nämlichen Ergebnis kam ein amerikanisches Komitee (the Vitamin B_1-subcommittee of the Accessory Food Factors Committee of the Medical Research Council) auf Grund von Kontrollen in mehreren Laboratorien.

Die von v. FELLENBERG angegebene Methodik wurde mit gewissen Modifikationen befolgt. Vor Oxydation des Aneurins zu Thiochrom wurden nach Vorschrift von HARRIS und WANG 3 ml Methylalkohol zugesetzt, um die Empfindlichkeit gegen Überschuss des Oxydationsmittels herabzusetzen.

Abweichend von diesen Autoren wurden die Ablesungen nicht in Proberöhrchen vor der Analysenquarzlampe vorgenommen, sondern es wurden zwei planparallele Küvetten verwendet, in welche das Licht senkrecht gegen die Flüssigkeitsoberfläche einfiel. Hierdurch gewinnt man im Gegensatz zum Reagenzrohr Vergleichsflächen mit gleichmässiger Lichtverteilung; auch kann man durch Abschirmung des Lichtbündels unmittelbar über den Küvetten die Eigenfluoreszenz des Glases ausschliessen.

Die beste Farbgleichheit bei Ablesung wurde erhalten, wenn man die O-Probe mit nur $^3/_4$ der beim Hauptversuch angewendeten Menge Substratextrakt versetzte. Die wahrscheinliche Erklärung dafür liegt darin, dass beim Hauptversuch die übrige Substanz, welche Fluoreszenz gibt, bei Zugabe von K_3FeCN_6 teilweise zusammen mit dem Aneurin oxydiert wird.

Eine irgendwie sichere Zahl für die Fehlergrenze der Methode bei

sorgfältigen Analysen lässt sich nicht angeben, doch dürfte der Wert bei ± 10—20% liegen. Die Fehler liegen indes vorwiegend in negativer Richtung, infolge unbefriedigender Extraktion, Spaltung und Oxydation sowie ausserdem wegen Vorextraktionsverlusten. Sowohl zu hohe wie zu niedrige Werte können entstehen durch Fehler bei der Herstellung der Thiochrom-Standardlösungen und bei der Ablesung. Die grösste Schwierigkeit liegt in der Erzielung exakter Farblosigkeit, da völlige Ausschaltung des Eigenfluoreszenztones bei den aschereichen Mehlfraktionen trotz der Vorextraktionen nur schwer zu ermöglichen ist. Wenn eine leichte Differenz in der Eigenfluoreszenz nicht zu grosser Unsicherheit in der Beurteilung führen soll, bedarf es langer Ablesungsgewohnheit. Daraus dürfte hervorgehen, dass zur Gewinnung einigermassen exakter Werte eine Mehrzahl von Bestimmungen erforderlich ist. In dem unten mitgeteilten Analysenprotokoll stellt die Vitaminzahl das Mittel aus gefundenen Werten dar. Die Anzahl Bestimmungen bei jeder Fraktion schwankte zwischen 2 und 6, je nachdem ob die Konstitution des fraglichen Produktes von besonderer Art oder der von anderen ähnlich war.

Die *Aschenwerte* wurden durch Veraschung auf trockenem Wege mit Magnesiumacetat erhalten.

Die *Proteinbestimmungen* wurden nach der Kjeldahlmodifikation von H. Lundin und J. Ellburg (1928) ausgeführt.

Die *Fettbestimmungen* wurden mittels Extraktion im Soxhletapparate, die *Feuchtigkeitsbestimmungen* mit Brabenders Halbautomaten vorgenommen.

Die Backversuche.

Da eine grosse Zahl Mehlfraktionen so beschaffen sind, dass es praktisch unmöglich ist, aus ihnen allein ein Probebrot zu backen, wurde statt dessen so verfahren, dass man ein Mehlgemisch aus 85% eines normalen Weizenmehles und 15% desjenigen Passagemehles herstellte, welches untersucht werden sollte. Infolgedessen ergibt das Backresultat die verbessernde oder verschlechternde Wirkung des Passagemehles auf das Standardmehl, und aus den gefundenen Ergebnissen lässt sich eine Vorstellung über die eigene Backfähigkeit eines jeden Passagemehles gewinnen.

Die Backversuche wurden nach der für schwedische Verhältnisse ausprobierten Backmethode (Å. Åkerman und J. Jacobsson 1936)

vorgenommen. Als Norm der Backfähigkeit eines Mehles dient das vom Mehl gelieferte Brotvolumen. Das auf 100 g Mehl bei 15% Wassergehalt umgerechnete Brotvolumen heisst das *spezifische Brotvolumen.*

Es verdient erwähnt zu werden, dass das spezifische Brotvolumen nicht allein zeigt, dass man beim Backen in der Praxis ein Brot von bestimmtem Volumen erhalten kann, sondern auch, dass die Backmethode so abgepasst ist, dass das spezifische Brotvolumen ein Wertmesser für die Mehrzahl derjenigen Eigenschaften eines Mehles ist, welche unter dem Begriffe der Backfähigkeit zusammengefasst werden. So zeigt die Praxis, dass in Schweden ein Weizenmehl eine Backfähigkeit haben muss, die einem spezifischen Brotvolumen von 650 ml entspricht, um den praktischen Anforderungen in Bäckerei und Haushalt zu genügen. Spezielle Brottypen setzen erheblich höhere Backfähigkeit voraus.

Analysenprotokoll.

	Ausbeute %	Aschengehalt %	Protoingehalt %	Fettgehalt %	Spez. Brotvolumen ml.	B_1-Vitamingehalt γ %
Winterweizen	65	1,53	10,7	2,05	—	333
Sommerweizen	35	1,55	10,9	2,11	—	311
Mehl 1. Schrotung	2,7	0,39	7,6	1,21	655	50
» 2. »	3,1	0,46	10,2	1,34	655	80
» 3. »	3,5	0,63	11,5	1,57	670	150
» 4. »	1,4	0,95	13,1	2,43	655	300
» 5. »	1,4	1,27	12,4	2,59	—	310
» 6. »	1,1	1,68	12,7	3,19	505	475
Dunst 6. »	—	2,83	15,7	6,18	370	1080
Mehl Kleiestreifen	0,7	1,94	13,3	4,13	470	905
Dunst »	—	3,20	16,2	7,07	330	1760
Mehl Sortiersichter 1., fein	4,7	0,44	9,9	—	615	90
» » » griffig	6,8	0,45	10,0	—	660	90
» » 2.	0,1	0,78	10,8	1,92	600	380
Mehl 1. Grobgriessauflösung, fein	4,1	0,44	8,0	—	620	105
» » » griffig	4,4	0,41	9,0	—	610	115
» 1. Feingriessauflösung, fein	7,4	0,37	8,4	—	610	60
» » » griffig	7,2	0,38	9,8	—	580	70
» 2. Griessauflösung, fein	2,8	0,53	8,8	1,61	600	180
» » » griffig	—	0,68	10,3	1,73	560	265
» » » grobgriffig	—	1,12	10,9	2,56	540	705
» 3. » fein	0,3	1,51	11,2	4.33	520	1300
» » » griffig	—	2,87	16,4	6,69	350	2400
» 1. Übergang, fein	0,7	0,99	10,3	2,93	610	710
» » » griffig	2,1	1,88	13,3	4,39	440	1250
» 2. » fein	0,3	1,00	11,5	2,80	600	640
» » » griffig	0,4	1,32	12,4	3,26	430	840
Mehl 1. Dunstausmahlung, fein	5,3	0,36	9,0	1,07	630	55
» » » griffig	1,7	0,40	10,1	1,20	630	100
» 2. » fein	2,4	0,41	9,8	1,15	670	130
» » » griffig	0,7	0,42	10,4	1,28	645	150

	Ausbeute %	Aschengehalt %	Proteingehalt %	Fettgehalt %	Spez. Brotvolumen ml.	B_1-Vitamingehalt γ %
Mehl 3. Dunstausmahlung, fein	5,0	0,45	10,1	1,20	650	115
» » » griffig	—	0,48	10,9	1,30	575	165
» 4. » fein	2,0	0,55	10,5	1,42	570	240
» » » griffig	—	0,53	11,0	1,53	580	225
» 5. » fein	0,9	0,98	11,5	2,36	550	755
» » » griffig	—	0,92	11,7	2,62	580	630
» 6. » fein	0,6	0,92	11,5	2,58	580	610
» » » griffig	1,4	0,96	12,7	2,95	530	720
» 7. » fein	0,5	1,09	13,6	2,58	570	560
» » » griffig	1,1	1,05	13,4	2,78	550	660
» 8. » fein	0,4	1,06	12,8	2,69	550	800
» » » griffig	1,2	1,51	13,2	3,65	440	1100
» 9. » fein	1,0	1,73	13,5	3,82	440	1180
» » » griffig	0,6	2,88	15,3	5,39	330	1450
» 10. » fein	0,5	2,26	14,0	4,86	410	1250
» » » griffig	0,2	3,15	15,7	6,49	320	2020
» 11. » fein	0,5	3,17	15,3	6,76	340	2090
» » » griffig	1,6	3,80	15,7	7,35	310	1940
» Koppen, fein	0,3	0,93	10,0	2,72	570	415
» » griffig	—	1,22	12,1	3,52	500	880
» Filter 1	0,3	1,04	12,3	2,34	640	515
» » 2	0,1	0,36	9,7	—	—	70
Keim 3. Griessauflösung	<0,1	4,33	18,0	5,75	340	730
» 1. Übergang	0,1	4,18	18,8	9,90	310	1420
» 2. »	<0,1	4,54	15,9	5,10	330	685
Kleie 6. Schrotung	10,6	6,13	11,7	3,51	380	445
» Kleiestreifen	4,7	4,88	12,2	4,77	330	535
» 10. Dunstausmahlung	0,8	4,33	12,3	5,46	—	650
» 11. »	1,3	3,92	13,1	6,07	280	685

B_1-Vitamin-, Aschen- und Proteingehalt sowie spez. Brotvolumen sind auf 15 % Feuchtigkeit, Fettgehalt auf Trockensubstanz bezogen.

Die Passagen, für welche Ausbeutewerte nicht angegeben sind, sind weiterer Vermahlung unterworfen.

Einige Passagen sind wegen ihrer Grösse in parallelgeschalteten Sichtern gesichtet, wodurch mehrere Fraktionsgruppen gleicher Art anfallen. Für diese Fraktionen sind nicht die Einzelwerte eingetragen, sondern die Durchschnittswerte. Die Einzelwerte sind aus dem Stapeldiagramm (Fig. 6) ersichtlich.

Nachstehend geben wir eine Kontrolle auf der Analysenresultate durch Zusammenrechnen der Produkte von prozentualer Ausbeute der Fraktion und deren Asche-, Protein- resp. Vitamingehalt. Als Vergleichszahlen werden Werte für den vermahlenen Weizen angegeben, bezogen auf den Bruchteil von Winter- und Sommerweizen im Gemisch.

	Asche	Protein	Vitamin B_1
Weizen.....	1,54	10,8	325
Fraktionssumme.....	1,55	10,6	321

Die Werte stimmen gut überein, was sich zum Teil aus der grossen Anzahl Passagen erklärt, durch welche vereinzelte Bestimmungsfehler ausgeglichen werden. Hinsichtlich der Fettwerte konnte eine solche Kontrolle nicht durchgeführt werden, weil Fettbestimmungen nicht an allen Passagen vorgenommen wurden.

Erörterung des Verhältnisses zwischen B_1-Vitamingehalt und Aschen-, Protein- sowie Fettgehalt der verschiedenen Mehlpassagen.

I.

Wird bei der Vermahlung eine bestimmte Mehlausbeute angestrebt, so wählt man in erster Linie Passagen mit geringem Aschengehalt; ein eventueller Zusammenhang zwischen *Aschen- und Vitamingehalt* verschiedener Fraktionen ist deshalb von grossem Interesse.

Aus der graphischen Darstellung in Fig. 2 geht hervor, dass im ganzen der Aneuringehalt mit dem Aschengehalt bis zu ca. 3 % hinauf ansteigt. Hierauf sinkt er und erreicht für die Kleiefraktionen Werte, die die durchschnittliche Aneurinkonzentration des Kornes unbeträchtlich übersteigen.

Aus der Prüfung des Verhaltens der verschiedenen Vermahlungssysteme zueinander ergibt sich unmittelbar, dass Schrotmehle beträchtlich niedrigere Vitaminwerte aufweisen als Dunst- resp. Griessmehl mit demselben Aschengehalt, was bereits früher von SCHULERUD und seinen Mitarbeitern (1941) beobachtet wurde. Die Erklärung hierfür ist wohl darin zu suchen, dass die geriffelten Walzen der Schrotungsserie ein wenig feinverteilte Kleie ablösen, welche den Gehalt an Asche verhältnismässig stärker erhöht als den an Vitamin.

Anfangs gehen Griess- und Dunstserien gut zusammen. Zwischen Aneurin- und Aschengehalt lässt sich ein nahezu linearer Zusammenhang bis zu ca. 1 % hinauf feststellen. Für die beiden weiteren Griessfraktionen bleibt dieser Zusammenhang im wesentlichen bestehen, dagegen beginnt die Asche in der Dunstserie verhältnismässig rascher zu steigen. Dies erklärt sich bei Betrachtung des Vermahlungsdiagrammes, aus welchem hervorgeht, dass u. a. die verhältnismässig aschereichen Übergänge von der sechsten und siebenten Schrotung zu weiterer Vermahlung in die hinteren Dunstausmahlungen gelangen.

Schlussfolgerungen.

1. Aus dem Aschengehalte einer Mehlfraktion lässt sich nichts Sicheres über ihren Vitamingehalt ableiten.

2. Innerhalb jeder Vermahlungsserie entspricht ein höherer Aschengehalt praktisch immer einem höheren Vitamingehalt. Eine Ausnahme bilden die Kleiefraktionen, bei welchen geringerer Aschengehalt einer höheren Vitaminkonzentration entspricht. Dieser Befund deckt sich durchaus mit dem älteren von PULKKI und PUUTULA (1941) u. a., dass die Samenhülle relativ vitaminarm ist.

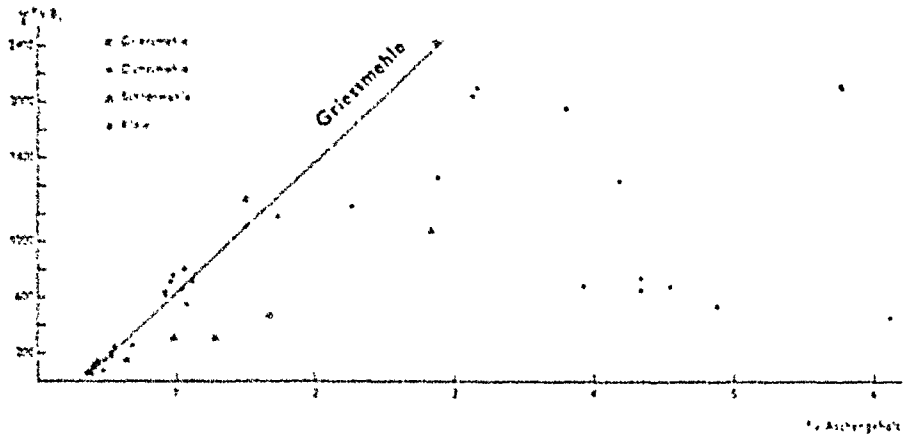

Fig. 2. Verhältnis zwischen B_1-Vitamin- und Aschengehalt der verschiedenen Mehlfraktionen.

II.

ZIEGLER (1943) betont, dass bei Mehlausbeuten zwischen 74 und 100% sehr hochgradige Korrelation zwischen Gehalt *an Protein und an B_1-Vitamin* bestehe.

Wie aus Fig. 3 hervorgeht, gilt in dieser Hinsicht weder für die einzelnen Passagen noch für die verschiedenen Vermahlungsserien ein einfacher Zusammenhang. Als Beispiel sei angeführt, dass bei ca. 16% Protein der Vitamingehalt zwischen 685 und 2400 γ% wechselt. Im ganzen entspricht jedoch höherer Proteinzahl ein höherer Vitamingehalt. Doch besagt dies nicht, dass ZIEGLERS oben erwähnte Beobachtung fehlerhaft sei, denn er befasst sich nicht mit Passagen, sondern mit prozentualen Mehlausbeuten. In einem solchen Falle kommt es darauf an, wie man zwecks Erzielung der fraglichen Ausbeute die Passagen auswählt.

Aus dem Proteingehalt einer Mehlfraktion kann man nichts Sicheres über ihren Vitamingehalt ableiten.

III.

PULKKI und PUUTULA (1941) haben gefunden, dass ein nahezu linearer Zusammenhang zwischen *Rohfett und Vitamin* B_1 besteht,

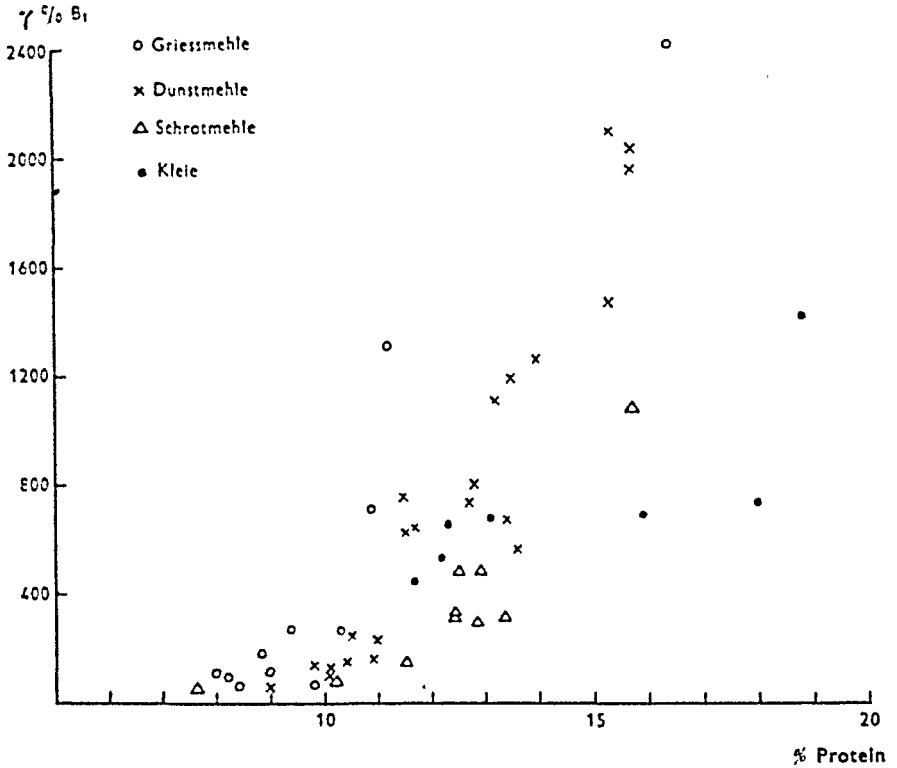

Fig. 3. Verhältnis zwischen B_1-Vitamin- und Proteingehalt der verschiedenen Mehlfraktionen.

der beträchtlich besser ist als der zwischen Asche und Vitamin B_1. Sie arbeiteten mit einer Serie Passagegemische mit steigendem Aschengehalt anstatt mit einzelnen Passagen, was die Ergebnisse von der Wahl der angewendeten Fraktionen abhängig macht.

Aus Fig. 4 ergibt sich ein Zusammenhang zwischen Fett und B_1, der im ganzen mit dem zwischen Asche und B_1 übereinstimmt. Die Beziehung nähert sich jedoch der Geraden mehr, als das bei der Asche der Fall war. Der Zusammenhang ist nicht einfacher Art, vielmehr entspricht jeder Vermahlungsserie eine Linie mit anderem

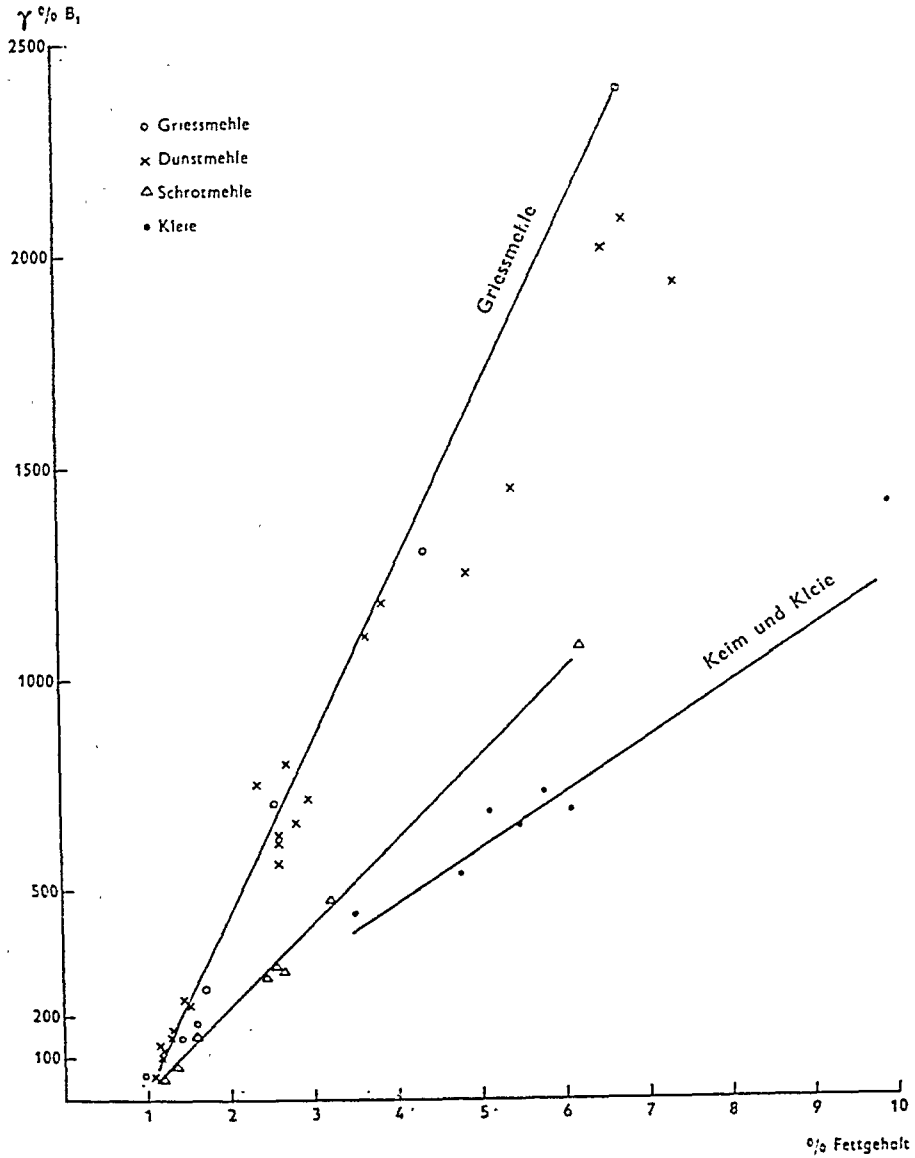

Fig. 4. Verhältnis zwischen B_1-Vitamin- und Fettgehalt der verschiedenen Mehlfraktionen.

Winkelkoeffizienten als bei anderen Serien. Griess- und Dunstserie stimmen im Anfang gut überein. Die Abweichungen bei den Mehlen der hinteren Dunstausmahlungen dürften sich ebenso erklären wie bei der Diskussion des Verhältnisses Asche : B_1. Am bemerkenswertesten ist, dass die Kleietypen und Keimfraktionen zusammen eine einigermassen gerade Linie ergeben, die gegen denselben Punkt kon-

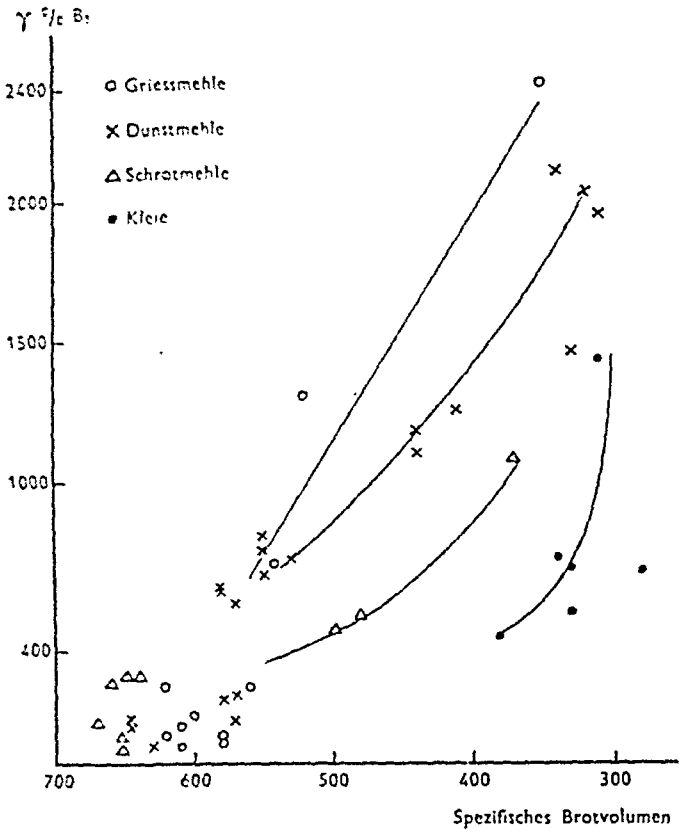

Fig. 5. Verhältnis zwischen B_1-Vitamingehalt und spezifischem Brotvolumen der verschiedenen Mehlfraktionen.

vergiert wie die anderen. Der Verlauf der Schrotungsserie scheint auf eine Mischung von Griess und Kleie hinzudeuten.

Schlussfolgerungen.

1. Aus dem Fettgehalte einer Mehlfraktion lässt sich kein sicherer Schluss auf ihren Vitamingehalt ziehen.
2. Innerhalb jeder Vermahlungsserie entspricht höherer Fettgehalt einem höheren Vitamingehalte.

Die oben angestellten Vergleiche lassen erkennen, dass die B_1-Einlagerungen im Korn von so spezifischer Art sind, dass man direkte Ausdrücke für die Aneurinkonzentrationen der verschiedenen Fraktionen zur Zeit lediglich aus direkt darauf gerichteten Analysen gewinnen kann.

IV.

Fig. 5 zeigt, dass zwischen *spezifischem Brotvolumen und* B_1-*Vitamin-Gehalt* kein einfacher Zusammenhang besteht.

Man kann jedoch deutlich drei Systeme unterscheiden, nämlich die Dunst- und Griessmehle, die Schrotmehle und endlich die Kleiefraktionen. Bei gleichem Vitamingehalt schädigen die letztgenannten die Backfähigkeit in viel höherem Grade als die übrigen.

Die Untersuchungen zeigen, dass, wenn man durch höhere Ausmahlung den Vitamingehalt des Mehles bis auf einen gewissen Wert erhöhen will, die geringste Schädigung der Backfähigkeit erzielt wird, wenn man Mehl der Griessauflösungs- und Dunstausmahlungsserien auswählt und die Kleiefraktionen vermeidet.

Diskussion über Untersuchungen von Keim und Kleie.

Bei der Vermahlung wird der Weizenkeim auf Grund seiner physikalischen Eigenschaften zum Teil abgetrennt, man erhält ihn in Gestalt einer flachgewalzten Scheibe. Der Literatur zufolge soll er 2 bis 3 Gewichtsprozent des gesamten Kornes ausmachen. Sofern die gewonnene kleine Keimscheibe für die Zusammensetzung des Keims repräsentativ ist, muss jede einzelne unverletzte Scheibe dem genannten Prozentbetrage des Kornes entsprechen. Um festzustellen, ob dies zutrifft, wurden 300 Körner und ebenso viele „Mühlenkeime" gewogen. Dabei ergab sich ein Mittelwert von 0,6% des Korngewichtes. Demnach kann der beim Mahlen erhaltene Keim nur ein kleiner Teil des natürlichen sein, derjenige nämlich, welcher am festesten zusammenhängt. Nach KUTHY (ref. ZIEGLER 1943) besteht im Keim eine zwischen 1200 und 12000 γ% variierende Differenz der Konzentration von B_1-Vitamin. Der höchste Gehalt findet sich im Scutellum (zunächst dem Kerne gelegen). In unserem Falle wurde der Gehalt an B_1 zu 1400 γ% bestimmt. Die Keimausbeute betrug nur 0.1% eines reinen Produktes. Der quantitativ grösste und qualitativ wertvollste Anteil des Keimes ist offensichtlich in andere Passagen übergegangen. Darauf dürfte sich erklären, warum das Mehl der 3. Griessauflösung als Endprodukt in der Griesserie den höchsten Vitaminwert aufweist, denn eben in dieser Serie wird der Keim abgestossen. Hier reichern sich also Keimreste mit hohem Vitamingehalte an. Auch in der Dunstserie wird sicherlich ein Teil des Keimes vermahlen. In die hinteren Dunstausmahlungen wird ferner der Übergang von der 3. Griessauflösung zu weiterer Vermahlung überführt. In Übereinstimmung damit finden wir bei den 10. und 11. Dunstmehlen sehr hohe Werte. In SCHULERUDS Fraktionsanalyse wird keine Zahl für den B_1-Gehalt des Keimes angeführt, jedoch betont, dass die Mehle der hinteren Dunstausmahlungen sich Werten nähern, welche selbst der reine Keim kaum aufweisen kann.

Aus den Analysenresultaten geht hervor, dass unter den Kleie-

fraktionen die vom 6. Schrot am ärmsten an Vitaminen und an Fett, dagegen am reichsten an Salzen ist. Die Dunstkleie mit grösserer Beimischung von Aleuronschicht zeigt den höchsten Gehalt an B_1 und Fett. Eine Mittelstellung nimmt „Feinkleie" (Kleiestreifen) ein. Kleie der 6. Schrotung ist repräsentativ für die Zusammensetzung der Samenhülle, doch enthält auch diese Passage ansehnliche Mengen Endosperm und Aleuronschicht, so dass der Vitaminwert höher liegt als für die eigentliche Samenschale.

Diskussion über B_1-Vitamingehalt und Ausbeute.

Ausmahlungsprozent	A. Die Passagen ausgewählt nach steigendem Aschengehalt			B. Die Passagen ausgewählt nach steigendem Gehalt an Vitamin B_1. (Kleiepassagen ausgeschlossen.)		C. Vergleichswerte nach Ziegler	
	γ % B_1	Proz. B_1 vom Ganzen	Proz. Diff.	γ % B_1	Proz. B_1 vom Ganzen	Ausmahlungsprozent	Proz. B_1 vom Ganzen
25	15,3	4,75		14,5	4,52		
			1,53				
30	20,1	6,28		18,5	5,78		
			2,91				
40	29,5	9,19		27,7	8,66	40,1	6,3
			2,95				
50	39,0	12,1		38,5	12,0		
			5,0				
60	54,7	17,1		53,0	16,6	63,5	17,4
			4,1				
65	68,0	21,2		65,7	20,5		
			9,3				
70	97,8	30,5		88,4	27,6	70,45	24,5
			11,0				
75	133	41,5		125	39,1	74	31,2
			16,0				
80	184	57,5		184	57,5	81,04	56,3
			15,1				
82,5	233	72,6		233	72,6		
			5,4				
85	250	78,0					
			8,1				
90	276	86,1				92,48	88
			7,0				
95	298	93,1					
			6,9				
100	321	100					

Tabelle 1. Der Gehalt des Weizenmehles an Vitamin B_1 bei verschiedenen Ausmahlungsprozenten.

In obiger Tabelle sind nebst den eigenen Werten Vergleichszahlen, die aus ZIEGLERS Untersuchungen berechnet sind, angegeben. Die Tabelle ist in Fig. 7 graphisch dargestellt. Die Übereinstimmung ist im ganzen gut. Es kann weiter angeführt werden, dass nach SLATER und RIAL (1941) bei 70% Mehlausbeute 30% vom gesamten Vitamingehalte des Kornes gewonnen werden, was mit dem vorstehenden Ergebnis völlig zusammenfällt. Ähnliche Ziffern legen DROESE und BRAMSEL (1941) vor. Um die besten Bedingungen für

Vergleich mit älteren Untersuchungen zu gewinnen, sind nicht die absoluten Vitaminwerte angegeben, sondern diese auf den Vitamingehalt des verwendeten Weizens bezogen worden. Dieser weist nämlich bei verschiedenen Weizensorten eine ausgesprochene Diffe-

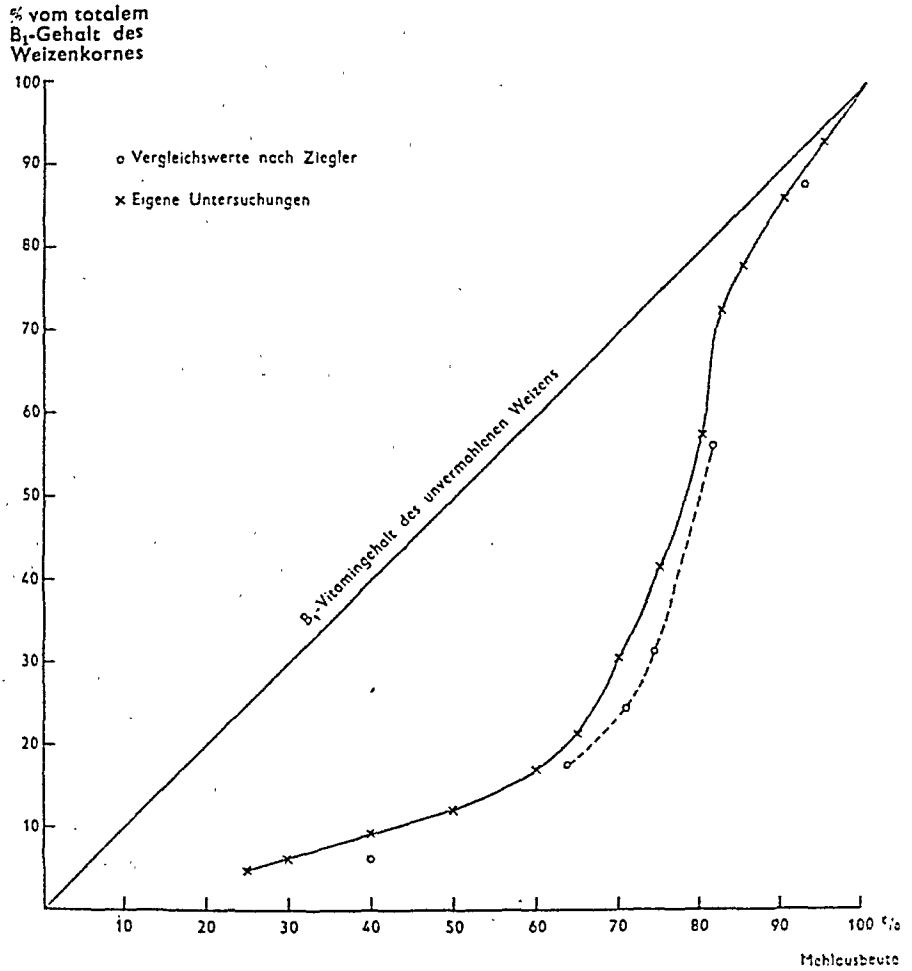

Fig. 7. B_1-Vitamingehalt bei verschiedenen Mehlausbeuten.

renz auf, abgesehen davon, dass die Wachstumsverhältnisse nicht vernachlässigt werden dürfen. Nach ZIEGLER geben JOHANSSON und RICH (1942) an, dass die Werte für amerikanischen Weizen zwischen 220 und 800 γ% variieren. SCHULTZ, ATKIN und FREY (1941) erhielten 560 γ% als Mittelwert für 39 Weizentypen. In unserem Falle lag die B_1-Konzentration ziemlich niedrig, bei nur 325 γ%, was möglicherweise zum Teil dadurch zu erklären ist, dass der

Weizen nicht von höchster Qualität war, weil u. a. seine Wachstumsbedingungen wegen anhaltender Dürre wenig günstig waren.

Aus Fig. 6 und 7 sowie Tabelle 1 geht hervor, wie ungleichmässig das Aneurin sich auf die Passagen verteilt und wie dies auf den Aneuringehalt bei verschiedenen prozentualen Mehlausbeuten einwirkt. So gelangen bei einer Ausbeute von 70% nur 30,5% der totalen Vitaminmenge mit in das Mehl. Erhöht man dagegen die Ausmahlung um 12,5% auf 82,5%, so gewinnt man 72,6%, d. h. auf diese 12,5% Mehl entfallen nicht weniger als 42% dieser Vitaminmenge. Wie die Backversuche zeigen, verursachen indessen diese nährungsphysiologisch äusserst wertvollen Fraktionen zwischen 70 und 82,5% eine ansehnliche Verschlechterung der Backfähigkeit, und darin liegt auch der Grund, warum sie früher aus allen feineren Weizenmehlsorten ferngehalten wurden. Erhöht man die Vermahlungsausbeute über 82,5% hinaus, so muss man wenigstens einen Teil reiner Kleiefraktionen dem Mehle beimischen. Diese besitzen aber wiederum eine im Verhältnis zum Vitamingehalte sehr hohe Aschenmenge (Fig. 2 und 6), und nimmt man sie mit in das Mehl auf, so verdirbt man also dessen haushaltstechnische Eigenschaften in viel höherem Grade, als es bis zu 82,5% hinauf der Fall ist.

Unter dem Gesichtspunkte der Vitaminversorgung wird mit Erhöhung der Ausbeute durch Beimischen reiner Kleiefraktionen zum Mehl wenig gewonnen, da diese im Mittel 497 γ% gegen 325 γ% des Weizens enthalten. Wichtigere Gründe für Beimischung von Kleie liegen in deren Reichtum an Salzen (u. a. Mg und Fe) sowie an Schlackenstoffen, welche auf Erhöhung der Peristaltik und der Sekretion des Darmes hinwirken.

Über Möglichkeiten, die B_1-vitaminreichsten Mehlfraktionen als Menschennahrung auszunutzen.

Sowohl Vitamin B_2 (Copping 1943) wie Vitamin E (Litzendorff, Schneider 1942) zeigen ähnliche morphologische Einlagerung im Weizenkorn wie Vitamin B_1, so dass diese „Schutznährstoffe" mit den höchsten Konzentrationen in denselben Passagen auftreten wie das Aneurin. Diese Passagen haben ausserdem höheren Gehalt an Fett und Eiweiss als das Endosperm (s. Analysenprotokoll S. 15) und machen deshalb die nahrungsphysiologisch wertvollsten Fraktionen aus. Ihre Nutzbarmachung als menschliches Nahrungsmittel scheint wünschenswert zu sein, falls man nicht den angloamerikanischen Weg einschlägt und synthetische Vitamine zusetzt.

Die bestehende Kriegslage hat dieser Frage zeitweilig eine unter ernährungsphysiologischem Gesichtspunkt glückliche Lösung gegeben, insofern als die gedrückte Lebensmittellage zu beträchtlicher Erhöhung der Mehlausbeute bei der Vermahlung Veranlassung gegeben hat. Hierbei sind die vitaminreicheren Passagen den zum Verkauf gelangenden Weizenmehlen zugeführt worden, jedoch nur zum kleineren Teile, weil diese anderenfalls haushaltstechnisch weniger tauglich geworden wären. Statt dessen hat man die vitaminreichsten Passagen den Roggensicht-[1] und Roggenschrotmehlen[2] beigemischt, welche dadurch einen sehr hohen Gehalt an Schutznährstoffen erhalten haben. Dadurch dass bei der Mehlzuteilung Roggen- und Weizenrationen getrennt werden, ist allen Konsumenten beim Einkauf ihrer Anteile eine Vitaminzufuhr gesichert worden, welche mehr ausgeglichen ist als vor dem Kriege. Nach dem Kriege muss das Problem wieder aktuell werden, wie man eine ausreichende Zufuhr von Vitamin B_1 zustande bringen kann. Höchstwahrscheinlich wird dann auf Verlangen der Konsumenten eine Rückkehr zu den weisseren Mehltypen erfolgen, welche die Vitaminversorgung des

[1] Schwed.: „Rågsikt och samsikt", Engl.: „Sifted rye flour".

[2] Schwed.: „Sammalet rågmjöl", Engl.: „Rye whole meal".

Einzelnen im wesentlichen von der Menge verzehrten Roggenbrots abhängig macht. In dieser Lage erscheint es als in vorderster Linie wünschenswert, die nährungsphysiologisch besten Passagen nicht wie früher zusammen mit der reinen Kleie als Futtermittel zu verwenden, sondern sie eventuell weiterhin dem Roggensicht- oder Roggenschrotmehl beizumischen.

In Schweden ist von jeher sowohl Weizen- wie Roggenmehl verzehrt worden, was eine derartige Lösung ermöglichen und erleichtern dürfte. Die Handelsvermahlung[1] in unserem Lande betrug vor dem Kriege ca. 62% Weizenmehl, 23% Roggensicht und 15% Roggenschrotmehl.

Vor dem Kriege wurden vom Weizen durchschnittlich 62% als Weizenmehl entnommen. Die allgemeine Auffassung war, dass die Backfähigkeit bei weiter gesteigerter Ausbeute wesentlich abnehme, was auch die vorgenommenen Backversuche bestätigten. Weizenmehl der Ausbeute zwischen 62 und 75% konnte in gewissen Fällen in billigeren Weizenmehlqualitäten vorkommen. In grösstem Umfange kam es jedoch zur Verwendung als Zusatzmehl zu Roggensichtmehl. Weizenmehl von höherer Ausbeute als 75% wurde nur in geringem Umfange und in dem Masse entnommen, wie die Mühlen Absatz für billigere Typen von Roggenschrotmehl hatten, die besonders als sogenanntes „Streumehl" in der „Knäckebrot"-Fabrikation Anwendung fanden.

Bei Verwendung von höher als auf 65% ausgemahlenem Weizenmehl traten besonders in Jahren mit keimgeschädigter Ernte[2] Übelstände im Haushalt beim Backen von Pfannkuchen, Anrühren von Suppen und Saucen u. s. w. auf. Dass auch die schwedischen Waldarbeiter Schwierigkeiten hatten, aus einem solchen Weizenmehl ihre ganz spezielle Mehlspeise[3] herzustellen, ist in Fachkreisen allgemein bekannt.

Diese Tatsachen sprechen dafür, dass, wenn den praktischen Anforderungen an die Mehlqualitäten genügt werden soll, zu einer prak-

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[1] Hiermit ist die durchschnittliche Konsumtion der schwedischen Bevölkerung erfasst, ausgenommen die Konsumtion derjenigen Teile der Bevölkerung, die ihr Mehl durch Vermahlung ihres eigenen Weizens in den sog. Lohnmühlen erhalten.

[2] Dies hängt zusammen mit dem höheren Gehalt an α-Amylase in der Randschicht des Weizenkornes nach beginnender Keimung.

[3] Schwedisch: „Kolbullar."

tisch bedeutsamen Steigerung des B_1-Gehaltes eine so grosse Erhöhung der Mehlausbeute beim Weizenmehl erforderlich sein würde, wie sie zur Zeit nicht durchführbar ist.

Vom Weizenmehl wird, wie bereits erwähnt, praktisch verlangt, dass es beim Backen ein Brot mit einem spezifischen Brotvolumen von wenigstens 650 ml gibt. An Roggensichtmehl sowie an Roggenschrotmehl stellt man keine so hohen Anforderungen, weil diese für Herstellung ganz anderer Brottypen als aus Weizenmehl dienen. So verwendet man das Roggenschrotmehl hauptsächlich nur für „Knäckebrot" sowie in gewissem Umfange für Roggenmehlgrütze. Aus Roggensichtmehl stellt man Kastenbrot[1] und Brotlaibe[2] her. Diese Brottypen sind kompakter und haben feinere Poren als Weizenbrot. Ihr spezifisches Brotvolumen beträgt nur etwa 300 ml. Erhebliche Quantitäten Weizennachmehl, welche, dem Handelsweizenmehl beigemischt, dessen Backfähigkeit zerstören würden (vgl. Fig. 5), können deshalb ohne diesen Nachteil dem Roggensichtmehl zugemischt werden. In gewisser Weise können sie sogar verbessernd auf die Backfähigkeit des Roggensichtmehles einwirken.

Nach unserer Ansicht sollten die oben erwähnten Möglichkeiten, die hinsichtlich des Aneurins wertvollsten Passagen nutzbar zu machen, näher geklärt werden. Eine derartige Untersuchung ist auch bereits im Gange und von schon gewonnenen Resultaten kann erwähnt werden, dass Zusatz von 50% Weizennachmehl, der Ausbeute von 65 bis 80 % entnommen (B_1-Gehalt ca. 776 γ% gemäss Tab. 1), den Gehalt an B_1 in einem der Ausbeute bis ca. 78% entnommenen Roggensichtmehl von ca. 225 auf ca. 500 γ% erhöhen würde.

Es dürfte möglich sein, in ähnlicher Weise das Roggenschrotmehl hinsichtlich des Vitamins zu verbessern. Wenn man diesem, welches einen B_1-Gehalt von ca 350 γ% hat, etwa 10% Weizenmehl beimischen würde, welches der Ausbeute zwischen 80 und 82,5% entnommen ist (B_1-Gehalt ca. 1940 γ% nach Tab. 1), würde der B_1-Gehalt auf ca. 500 γ% steigen. Erfahrungen aus den vergangenen Kriegsjahren sprechen für die Realisierbarkeit dieses Vorschlages. Das schon bisher sehr wertvolle „Knäckebrot" würde so eine noch ergiebigere Vitaminquelle werden als vorher.

[1] Schwed.: „Formbröd", Engl.: „Tin loaf".

[2] Schwed.: „Friskjutet bröd (t. ex. limpa, franskbröd)", Engl.: „Heard loaf".

Eine andere Möglichkeit ist, das Weizennachmehl der Ausbeute zwischen etwa 80 und 82,5% als ein besonderes Grützmehl in den Handel zu bringen. Die Bezeichnung Grützmehl schliesst natürlich nicht ein, dass die Anwendbarkeit sich auf das Kochen von Grütze beschränken soll (wobei man am besten gleiche Teile Haferflocken, Hartgriess, Roggenschrotmehl oder Reis zumischt). Auch verschiedenen Gerichten lässt sich dieses Grützmehl sehr wohl beimischen, beispielsweise Puddingen, Fleischklössen, Brei und Absud für Kleinkinder.

Täglicher Verbrauch von 50 Gramm dieser vitaminreichen Fraktionen würde u. a. die Zufuhr von Vitamin B_1 um nicht weniger als 1 mg erhöhen, d. h. um nahezu den gesamten Bedarf an B_1-Vitamin. Dadurch dürfte bei jeder einigermassen gemischten Kost die Gefahr auch leichtester B_1-Hypovitaminosen beseitigt werden.

Hierdurch erhält auch der zahnlose Teil der Bevölkerung, dem das harte Brot Schwierigkeiten bereitet, eine geeignete Konsumtionsware. Nicht zum wenigstens kann ein solches Grützmehl vermutlich für Kranke, Schwangere und Kinder, welche erhöhter Vitaminzufuhr bedürfen, von Wert sein. Da die Partikelgrösse die des gewöhnlichen Mehles nicht übersteigt, braucht man eine Reizwirkung auf empfindliche Schleimhäute nicht zu befürchten, wie sie bei kleiehaltigem Mehl (Roggenschrotmehl, Grahammehl) auftreten kann. Gegenüber dem Weizenmehl hat das Grützmehl wegen des erheblich höheren Gehaltes an Schlackenstoffen den Vorzug, nicht verstopfend zu wirken.

Zusammenfassung.

1. Es werden die Resultate der Analyse aller Fertigprodukte einer modernen Weizenmühle vorgelegt. Die Untersuchung erstreckt sich auf Gehalt der verschiedenen Vermahlungspassagen an Vitamin B_1, Asche, Protein und Fett sowie auf deren Backfähigkeit.

2. Es wird der Zusammenhang zwischen Gehalt an Vitamin B_1 und an Asche besprochen und dabei nachgewiesen, dass auf Grund des Aschengehaltes einer Mehlfraktion zwar nichts Bestimmtes über deren Vitamingehalt ausgesagt werden kann, dass indessen innerhalb jeder der drei Vermahlungsserien höherer Aschengehalt praktisch immer einem höheren Vitamingehalt entspricht. Mehl aus der Schrotungsserie hat geringeren Vitamingehalt als Griess- und Dunstmehl vom gleichen Aschengehalt. Für die Kleiefraktionen gilt, dass höherer Aschengehalt geringerem Vitamingehalte entspricht.

3. Innerhalb jeder Serie von Vermahlungsprodukten entspricht höherer Fettgehalt der Fraktion auch einem höheren Gehalt an Vitamin. Bei gleichem Fettgehalt in Dunst- und Griessmehl, Schrotmehl und Kleie hat das Dunst- und Griessmehl den höchsten Aneuringehalt, die Kleie den niedrigsten; das Schrotmehl zeigt einen dazwischen liegenden Wert.

4. Zwischen Gehalt an B_1-Vitamin und an Protein besteht kein erkennbarer Zusammenhang bei den einzelnen Fraktionen.

5. Durch Backversuche wird gezeigt, dass, wenn man durch höhere Ausmahlung den Vitamingehalt des Weizenmehles bis auf einen gewissen Wert erhöhen will, die geringste Schädigung der Backfähigkeit erzielt wird, wenn man Mehl der Griessauflösungs- und Dunstausmahlungsserien auswählt und die Kleiefraktionen vermeidet.

6. Es wird über die Vitaminverteilung bei verschiedenen prozentualen Mehlausbeuten berichtet. Bei Ausbeute von 0 bis 65 % werden lediglich 21,2 % der Vitamine nutzbar gemacht. 51 % der totalen Vitaminmenge des Weizens fallen innerhalb einer Mehlausbeute

zwischen 65 und 82,5%. Die Fraktionen zwischen 82,5 und 100% Ausbeute (die Handelsware „Kleie") enthalten 27% der gesamten Vitaminmenge. Auf den ernährungsphysiologischen Wert der Fraktionen zwischen 65 und 82,5% Ausbeute als menschliches Nahrungsmittel wird hingewiesen.

Eine solche Ausnutzung hat auch in grossem Umfange infolge der durch die Krisenlage veranlassten Vermahlungsbestimmungen der letzten Kriegsjahre stattgefunden.

Es wird auf folgende Möglichkeiten für die Nutzbarmachung dieser vitaminreichen Fraktionen als Menschennahrung auch nach dem Kriege aufmerksam gemacht:

a. Beimischung von ca 50% Weizennachmehl aus Fraktionen zwischen 65 und 80% Ausbeute zu dem Roggensichtmehl, wodurch der B_1-Gehalt dieses Mehles von ca. 225 γ% auf ca. 500 γ% erhöht werden würde.

b. Beimischung von 10% Weizennachmehl aus Fraktionen zwischen 80 und 82,5% Ausbeute zu dem Roggenschrotmehl, welches hauptsächlich zur Herstellung von „Knäckebrot" Verwendung findet. Der B_1-Gehalt dieses Mehles würde dadurch von ca. 350 γ% auf 500 γ% erhöht werden.

c. Alternative Verwertung von Weizennachmehl der Ausbeute zwischen 80 und 82,5% als besonderes Grützmehl, dessen praktische Anwendbarkeit besprochen wird. Sein B_1-Gehalt würde wenigstens 1940 γ% betragen.

Jedes der nach diesen Vorschlägen hergestellten Mehle weist einen im Verhältnis zu seinem Kaloriengehalte beträchtlichen Überschuss an B_1-Vitamin auf.

Summary.

1. The result of analysis of all ready products in a modern flour mill is given. The investigation comprises vitamin B_1, ash, protein and fat content of all the different mill products and their baking capacity.

2. The relation between vitamin B_1 and ash content is discussed. It is here pointed out that the ash content of a flour fraction gives no definite information as to its vitamin content. Flour from the breaking rolls contain less vitamins than flour from the middling reductions. For the bran the rule is that a higher ash content corresponds to a lower vitamin content.

3. In each series of mill products (break and reduction flour, and bran) a higher fat content corresponds to a higher vitamin content. When the fat content is the same, the reduction flour has the highest vitamin content, the bran the lowest and the break flour shows a value lying in between.

4. Between vitamin B_1 and protein content there is no obvious relation for the individual fractions.

5. Baking tests demonstrate that if it is a desire to raise the vitamin content of the wheat flour to a certain value, the baking strength of the flour will be less weakened if flour from the middling reductions is used for this purpose and the bran avoided.

6. The proportion of the vitamins at different percentual flour extractions is described. A flour, which is taken out to 65 % extraction, contains only 21.2 % of the vitamins. 51 % of the whole vitamin content of the wheat falls between 65 and 82.5 % flour extraction. The fractions between 82.5 % and 100 % extraction (i. e. the commercial product bran) contain 27 % of the total vitamin content.

It is pointed out that there is a high nutritive value in preserving the fractions between 65 and 82.5 % extraction for human consumption.

Government regulations during the last years of war have made for an extensive preservation of this kind.

Possibilities of using these vitamin-rich fractions even after the war are suggested:

a) An admixture of 50% pollard to siftet rye flour. If the pollard is taken out between 65 and 80% extraction, the B_1-content would be raised from 225 γ% to approx. 500 γ%.

b) An admixture of 10% pollard taken out between 80 and 82.5% extraction to the rye wholemeal, which is mainly used in the production of hard rye-bread. The B_1-content in this flour would then be raised from 350 γ% to 500 γ%.

c) Alternatively, the preservation of the pollard between 80 and 82.5% extraction as a special porridge-flour, whose uses in practice are discussed. The B_1-content of this flour would be approx. 1940 γ%.

Each of the flours produced in accordance with these suggestions thows a considerable vitamin surplus in relation to its calory consent.

Angeführte Literatur.

A report by the vit. B_1-subcommittee of the Accessory Food Factors Committee of the medical Research Council and Lister Institute, Biochem. J. 1943, *37*, 433.

A. M. Copping, Biochem. J. 1943, *37*, 12.

Droese-Bramsel 1941, Leipzig. Vitamintabellen der gebräuchlichsten Nahrungsmittel.

Th. v. Fellenberg, Mitteil. a. d. Gebiet d. Lebensmittelunters. u. Hyg. 1942, *XXXIII*, 232.

L. J. Harris and Y. Wang, Biochem. J. 1941, *35*, 1050.

H. Johansson and C. E. Rich, Cereal. Chem. 1942, *19*, 308.

J. Litzendorff und E. Schneider, D. Ernähr. 1942, *7*, 203.

H. Lundin, J. Ellburg, Svenska Bryggarför. Månadsbl. 1928, h. 5.

L. H. Pulkki und Puutula, Biochem. Z. 1941, *308*, 122.

A. Schulerud, A. Kanter, A. Erichsen, Nord. Med. 1941, *10*, 1477.

A. S. Schultz, L. Atkin and C. Frey, Cereal. Chem. 1941, *18*, 106.

E. C. Slater, E. J. Rial, Austr. chem. Inst. J. Proc. 1941, *8*, 71.

E. Ziegler, Mitteil. a. d. Gebiet d. Lebensmittelunters. u. Hyg. 1943, *XXXIV*, 128.

Å. Åkerman och J. Jacobsson, Sverig. Utsädesför. tidskr. 1936, *XXXVI*, h. 1.

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FROM THE DEPARTMENT OF PHYSIOLOGY, UNIVERSITY OF LUND, SWEDEN

MECHANICAL PROPERTIES OF CARDIAC MUSCLE

BY

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MALMÖ

1 9 4 4

To my wife

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Introduction

When investigating structure and function of muscle, experiments on their mechanical properties are of great importance. These properties have been experimented on in skeletal muscle much more so than in cardiac muscle.

A skeletal muscle as a whole is far too complicated a body of muscle fibres, tendons, vessels and connective tissue to be suitable for a detailed analysis of mechanical properties such as elasticity and viscosity, and this applies in a still higher degree to the heart. This fact has been pointed out especially by Buchthal and Lindhard (1939) who in their experiments on skeletal muscle worked with the single fibre only.

Many investigations concerning function and mechanical properties of the heart dealing with the organ as a whole have been published. It is, however, difficult to interpret these results with regard to characteristics of the muscle substance proper, and for a number of properties no information at all has been obtained. Strips of cardiac tissue some mm^2 in section and several mm in length cut from the wall of the ventricle are likewise unsuitable for an analysis of structural properties.

An attempt is made here to give qualitative and quantitative data regarding mechanical properties of cardiac muscle for the resting and the contracted substance. Hitherto the static properties only have been the object of investigation. Here the dynamic properties are also examined using methods previously applied to skeletal muscle by Gasser and Hill (1924) among others.

In the following section a short survey of previous investigations on mechanical properties of cardiac muscle is attempted.

At the end of the last century, investigations of Dreser (1888) and others inspired Otto Frank (1898) to a more detailed

study of the dynamics of the heart which he tried to correlate with experiments on skeletal muscle by Fick (1882) and v. Kries (1880) (1882). Frank investigated thoroughly contraction curves under isometric and isotonic conditions in both auricle and ventricle. He determined the degree of filling when the heart performed its maximal tension. Furthermore he investigated the varying stroke volume as a function of different diastolic pressures and of varying arterial resistance in rigid and elastic vessels. However valuable these experiments are for understanding the dynamics of circulation, they give no further information on viscous, elastic and structural properties of cardiac muscle. However, when correlating mechanical properties with dynamics of the heart as a whole, Franks investigations will be used as a basis.

Bohnenkampf and Ernst (1926) published the first quantitative data on the material constant of cardiac muscle at rest. They determined the elasticity modulus which is defined as the tension (in kg/mm^2) necessary to stretch a body to twice its length.

According to Bohnenkampf and Ernst, cardiac muscle follows Hooke's law i. e. the muscle should extend proportionally with loading, a fact that holds good for most inorganic isotropic elastic bodies. This assumption was in contrast to older observations by Dreser (1888) and Frank (1898) which concluded from pressure-volume diagrams that cardiac muscle behaved as skeletal muscle i. e. with increasing length a relatively higher load is necessary to induce equal elongation. Bohnenkampf and Ernst, also working on the total ventricle of the heart of a frog, calculated the elasticity modulus for cardiac muscle from a formula of Sacredote (1898) at 80—90 kg/mm^2, a value found probable by a comparison with a number of other elasticity moduli e. g. of wood, iron etc.

Wöhlisch and Clamann (1936) criticising these experiments stated that the value was in accordance with the elasticity modulus of a strongly stretched tendon. One has only to take a tendon and a strip of cardiac tissue of about the same cross-section and stretch them, to see that the former has a much higher elasticity modulus than cardiac muscle. Furthermore Wöhlisch and Clamann showed that Bohnenkampf and Ernst, in their estimation of elasticity modulus, did not reduce the intraventricular

pressure expressed in mm Hg to the unit of elasticity i. e. kg/mm^2. Their elasticity modulus, therefore, was 76.000 times too high. The corrected values thus become 0.00105—0.00118 kg/mm^2, values which approximate those found for skeletal muscle.

As the pressure-volume curves for the heart found by Bohnenkampf and Ernst deviate from those of all other observations, Wöhlisch and Clamann (1936) re-investigated the basis for these corrected values and found an elasticity modulus minimum of 0.00028—0.00044 kg/mm^2 and an elasticity max. of 0.0029—0.0044, values which, as far as the min. is concerned, lie far beneath those found by Bohnenkampf and Ernst. Wöhlisch and Clamann obtained these values in experiments on strips cut from the ventricle wall 7—8 mm long and 3 mm thick.

Other experiments with the whole ventricle using the same formula as Bohnenkampf and Ernst resulted in an elasticity modulus minimum value of 0.00018 and a maximum value of 0.0030, values which agree with those found from strips of heart muscle. Up to now these quantitative data on resting cardiac muscle are about all we have at our disposal. It is rather surprising that observations on the elasticity modulus of *contracted* cardiac muscle are entirely lacking. Furthermore, the above mentioned investigations concern static properties only while an analysis of dynamic properties has not yet been performed.

The purpose of this investigation is to procure new data concerning mechanical and structural properties of cardiac muscle. These will be compared with data from skeletal muscle to see how far the results found will fit in with modern contraction theories.

To obtain data on cardiac muscle comparable with those from recent experiments on skeletal muscle fibres, it is necessary also here to work on well-defined fibre bundles or individual fibres. Therefore, only parallel threaded muscle bundles 0.2—0.4 mm thick and several mm long from the cardiac ventricle of a frog are used.

Method

1. General Method.

The small muscle bundles (fig. 1.) can be dissected with little difficulty. The bundles are taken from both apex and base of the ventricle. The heart of the frog is used because its contraction period is sufficiently long to determine mechanical properties by the method employed here, which is not the case with the mammalian heart. Both Rana temporaria and esculenta are used.

The muscle bundles are prepared under a binocular microscope (enlarging about 20 times) with a fine plane-polished tweezer and a special scissors (fig. 2). Isolated or only slightly anastomosing

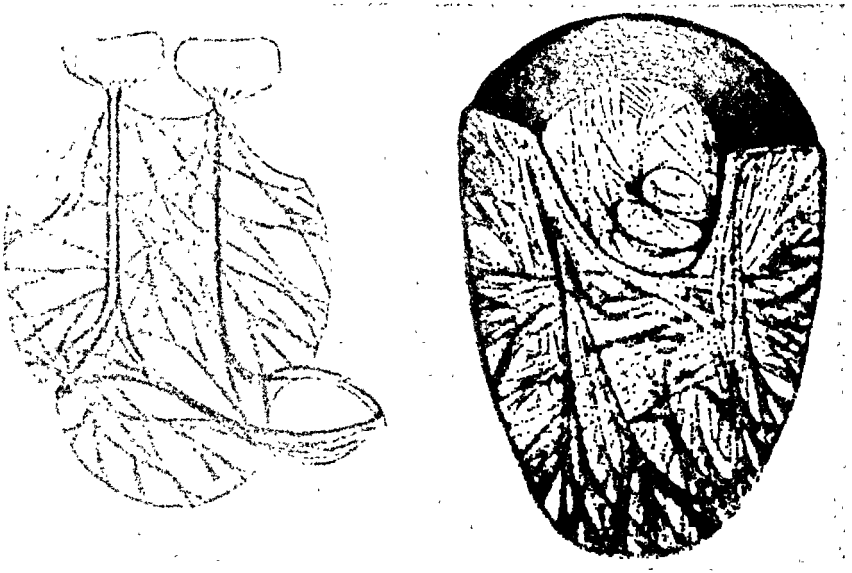

Fig. 1. Structure of cardiac auricle and ventricle of a frog (Gompertz 1884).

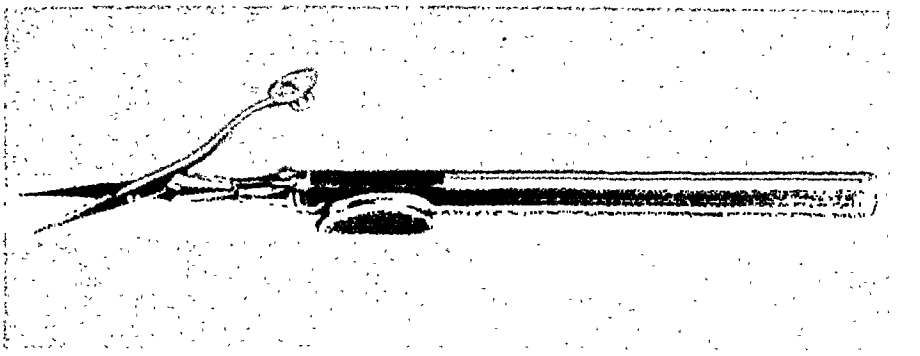

Fig. 2. Scissors for micro-dissection (B u c h t h a l).

bundles consisting of parallel lying fibres are used with uniform cross-section throughout their length. Long thin bundles could also be prepared from the auricle but as it is difficult to separate these from connective tissue the preparation from the ventricle is preferred.

The muscle is prepared in ice-cooled Ringer solution. pH in the solution after a stream of a mixture of CO_2 and 99 per cent O_2 had passed through was 7.3—7.4 and did not vary during the experiment. Determination of pH was performed by a glass electrode. To ensure a suitable colloid-osmotic pressure 6 per cent dialysed gum. arabic. was added. Control experiments on a bundle stretched about 50 per cent above the equilibrium length and performing 5400 contractions at room temperature in the course of six hours, showed the same strength of contraction and excitability at both start and finish of the experiment as well as an unchanged pH in the Ringer solution.

The majority of the experiments were performed at a *temperature* of 4—6°, the duration of contraction thereby being sufficiently increased to facilitate determinations of mechanical properties.

The preparation is *stimulated* with opening impulses from a Harvard coil. The tweezers holding the ends of the muscle bundle are used as stimulation electrodes and are electrically isolated from the apparatus. In each experiment controls ensure that no stimulus escape occurs. Stimuli are given at constant intervals by means of a motor-driven switch opening and closing the primary circuit of the Harvard coil.

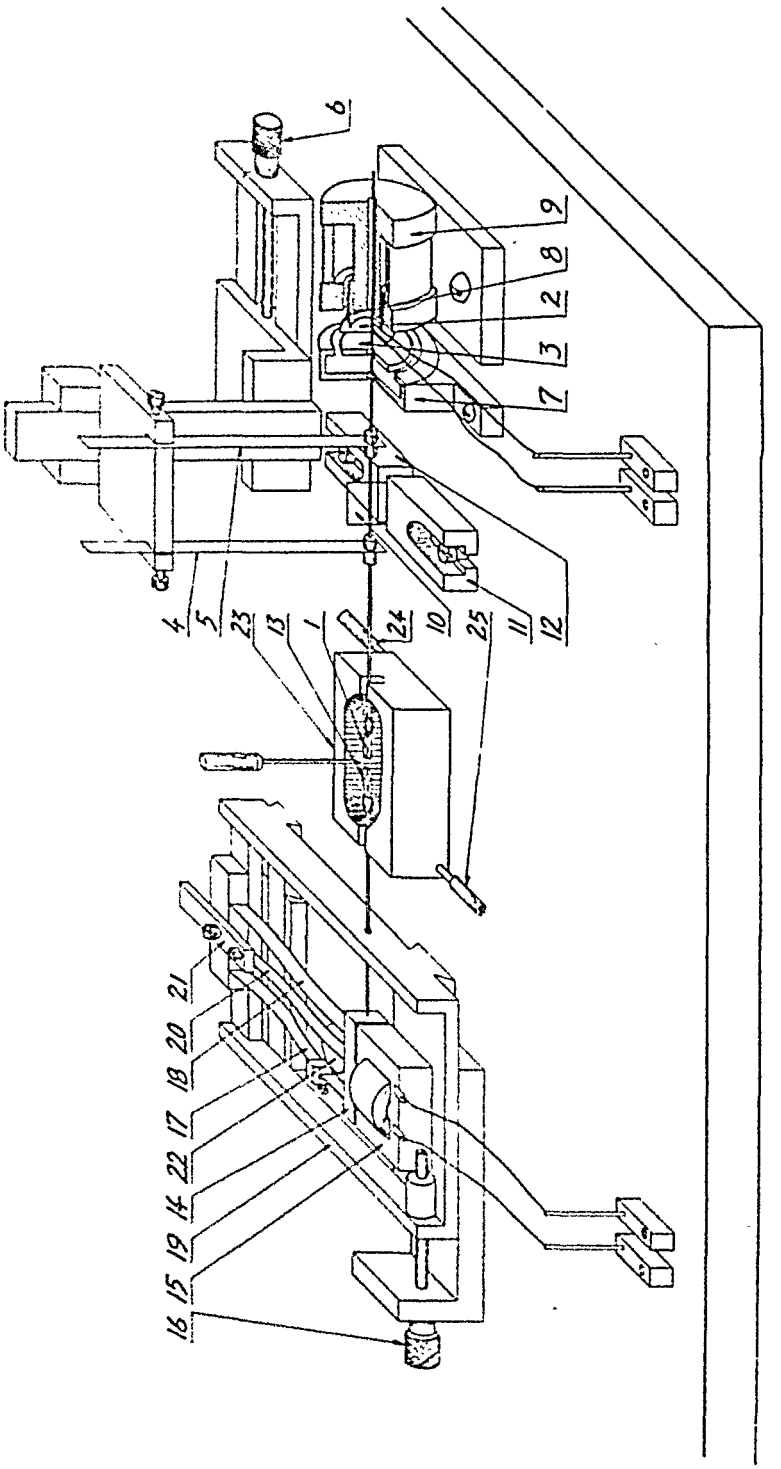

Fig. 3.

The *recording device* (fig. 3.) agrees in principle with the condensor-myograph devised by Buchthal (1942) in his experiments on skeletal muscle fibres. It consists of a condensor for recording tension containing two condensor plates, one fixed and one movable. When the muscle contracts or is stretched these plates approach each other so that their capacity is altered and this change is recorded by a high frequency circuit with suitable amplification.

The muscle is fixed between two micro-tweezers (1—2 mm diameter) made of plated phosphor-bronze. By pushing a ring over the prongs these close round the ends of the preparation and fix it. A loading test on the prongs by hanging weights on a hair shows that only when the weight exceeds 125 g does the hair slide off the prongs, a weight which is at least 100 times greater than the maximal load found in the experiments.

Fig. 3. Apparatus for registration of length-tension diagrams and measurements of dynamic stiffness.

(1) micro-tweezer.
(2) movable condensor plate in connection with (1).
(3) fixed condensor plate connected with grid of high frequency circuit.
(4) and (5) steel springs to keep tweezer (1) in position.
(6) micrometer screw by which distance between condensor plates (2) and (3) can be varied.
(7) screen for non-earthed condensor plate (3).
(8) coil attached to condensor plate (2).
(9) permanent magnet.
(10) thin mica disc gliding in oil between (11) and (12).
(11) and (12) brass blocks with adjustable distance for damping tweezer (1).
(13) micro-tweezer to hold other end of muscle fibre.
(14) iron bar holding micro-tweezer (13).
(15) electro-magnet.
(16) micrometer screw to vary distance between (1) and (13).
(17) and (18) strong steel springs to hold iron bar (14) in front of electro-magnet (15).
(19) brass frame holding (14), (15), (16), (17) and (18).
(20) steel spring which by tightening moves iron bar (14).
(21) movable metal plate which can be clamped in different positions and thus tighten spring (20).
(22) oil bath to damp movements of tweezer (13).
(23) chamber with Ringer solution.
(24) and (25) tubes for cooling water.

The movable condensor plate (2) is in direct contact with microtweezer (1) and is earthed. When muscle tension increases this condensor plate is pulled toward the fixed condensor plate (3). The microtweezer (1) is held by two steel springs 6 mm broad and 0.12 mm thick (4) and (5). They have an adjustable length and the stiffness of the recording system can be varied. Furthermore they bring about an absolutely parallel movement of the tweezer.

The sensitivity of the system mainly determined by the distance between the condensor plates — short distance, high sensitivity — can be regulated by moving the oscillating system with a micrometer screw (6) in a sledge of brass metal. The unearthed condensor plate (3) is shielded by a metal screen (7) from exterior capacity disturbances and stimulus escapes.

A coil (8) of 30 windings (wire diameter 0.1 mm) is attached to the condensor plate (2). The windings lie perpendicular to the tweezer axis. The coil moves freely in the air gap of a permanent magnet (9) (1000 Gauss). By leading a current through the coil, the magnet is made to attract or repel it. The purpose of this device is to ensure constant sensitivity in spite of the increase in muscle tension with its accompanying displacement of the movable condensor plate.

Tension during contraction is measured by capacity changes when the condensor plates approach each other. Tension in the resting fibre is determined by the size of the compensation current in the coil necessary to counteract the displacement of the movable condensor plate. The compensation current measured in mA must be calibrated to mg, the unit of tension. This calibration is performed by loading the movable tweezer and compensating its deviation by leading a current through the coil. Loading, which should act in the same direction as the tension in the fibre i. e. horizontally, is performed in the following way:

The tweezer is connected by a hair to the pointer of a precision balance and the scale near the tweezer increasingly loaded. The resulting deviation of the tweezer is compensated by leading current through the coil. The return of the tweezer to its starting point is controlled by a galvanometer (sensitivity 10^{-6} Amp.) connected to the output of the amplifier. The loads applied are 0.05, 0.1, 0.2, 0.3,

0.5 and 2 g. The compensation current rises linearly with loading. Calibration is performed for the different distances between the condensor plates which may occur in the experiments i. e. between 0.2 and 3 mm. Within this range the same compensation current is found for equal loadings. By measuring the distance between the attachment of the hair on the pointer and the edge of the balance (a) and the length of the weight arm (b) we get, when the load on the scale is termed A, the actual load on the tweezer $X = \frac{A \times b}{a}$. 1 mA is found to correspond to 8 mg. Calibration is also performed by means of a horizontal spring balance like that used by B u c h t h a l (1942). The results tally completely.

To produce *aperiodic movements* of the micro-tweezer (1) a thin mica disc (10) is attached to the tweezer shaft. This runs in a gap between two brass blocks (11. 12) the distance between which can be regulated. Oil of suitable consistency is placed in the gap between the metal blocks.

The other end of the muscle is held by a micro-tweezer (13) which is fixed to an iron bar (14) in front of an electro-magnet (15). The whole system, tweezer, iron bar and magnet with frame (19) is placed on a sledge which can be moved by the micrometer screw (16). In this way the distance between the tweezers can be varied i. e. the fibre can be stretched or relaxed. The length of the muscle can also be varied by altering the current in the electro-magnet (15). The iron bar (14) is fixed by two strong springs (17, 18) to a stable brass frame (19). By tightening a third spring (20) the iron bar can be moved backwards and forwards. Spring (20) is fixed to a movable metal plate (21) which by means of a screw can be clamped in different positions. By turning (21) the spring tenses and tries to shift the position of bar (14). Spring (20) is not firmly attached to the iron bar but moves in a slide in a brass block fixed on (14). Stiffness of spring (20) is about twice that of springs (17, 18). If current is led through the electro-magnet (15) the iron bar (14) tries to place itself so that springs (17, 18) are parallel with the direction of the magnetic field. Changes in length brought about by the current in the electro-magnet can be varied in size and rate by introducing suitable resistances and condensors in the circuit (fig. 4).

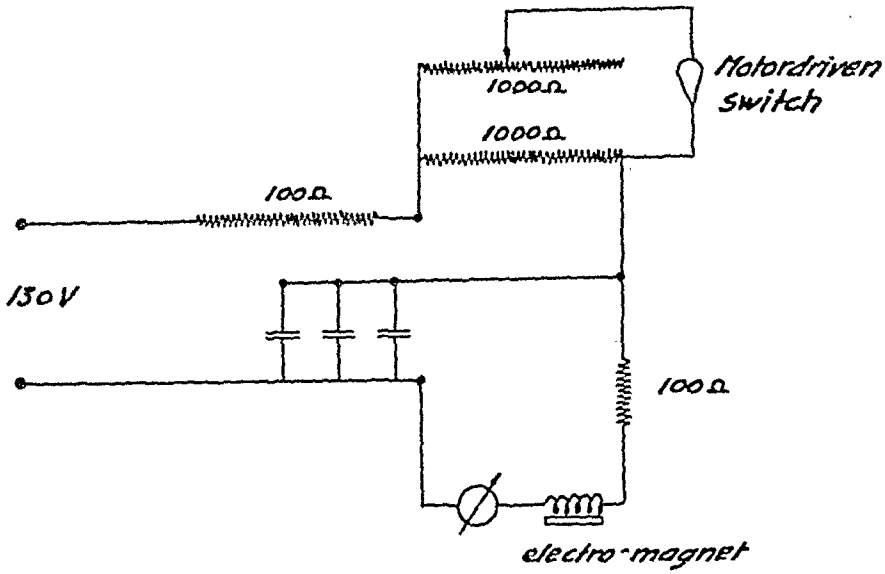

Fig. 4. Length variation device.

By correlating the current in the electro-magnet to the tweezer movements determined by a measuring microscope, it is possible to express variations in length by the current. As, however, this dependence would have to be determined for every new position of spring (20) the movements of the tweezer (13) in our experiment are read directly by means of an eyepiece micrometer. The movements of the iron bar micro-tweezer system (13, 14) are damped by means of a horizontal metal disc attached to spring (17) which slides in an oil bath of thick consistency (22).

The *length and changes in length* of the preparation are measured by a *Blix* measuring microscope (1895). This is slightly modified so that the entire arrangement can be placed on its foot plate. The measurable distance is 30 cm and accuracy with the enlargement used is 0.01 mm.

The experimental object is immersed in Ringer solution in chamber (23), made of plated brass with openings for the micro-tweezers in the short sides. A rectangular glass plate can be placed beneath the preparation to facilitate attachment of the fibre to the tweezers. The chamber is 6 mm deep with a volume of 5 ml. It can be emptied with a syringe via an opening at the bottom. To regulate tempera-

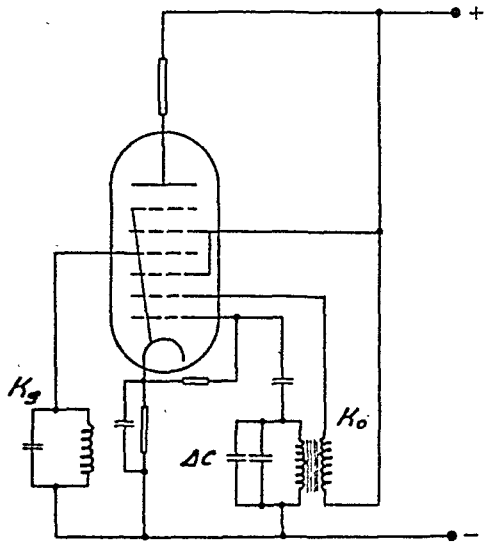

Fig. 5. High frequency circuit for measuring changes in capacity (explanation see text).

ture of the Ringer solution the chamber has a double bottom through which water of suitable temperature is led by tubes (24, 25). Two 10 litre bottles provide and collect the water for cooling. To ensure that it has the same temperature throughout, the mouth of a siphon tube is placed on a level with the lower edge of the ice layer in the water resevoir.

Temperature is controlled thermo-electrically. One element is placed in Ringer solution, the other in a Dewer vessel filled with ice-water. The thermo-current is measured by a galvanometer (Siemens and Halske 10^{-6} Amp.). 3 scale units correspond to 1° C. This cooling arrangement proved effective. Within a few minutes the Ringer solution was cooled from room temperature to 4—5° C and the temperature remained constant throughout the experiment provided that the siphon was kept in the position described. Temperature variations do not exceed $\pm$ 0.2° C.

High-frequency apparatus for measuring changes in capacity.

Tension variations in the muscle are transformed to changes in capacity in a small plate condensor (3 fig. 3). Changes in capacity are measured by a high frequency arrangement (fig. 5) the principle

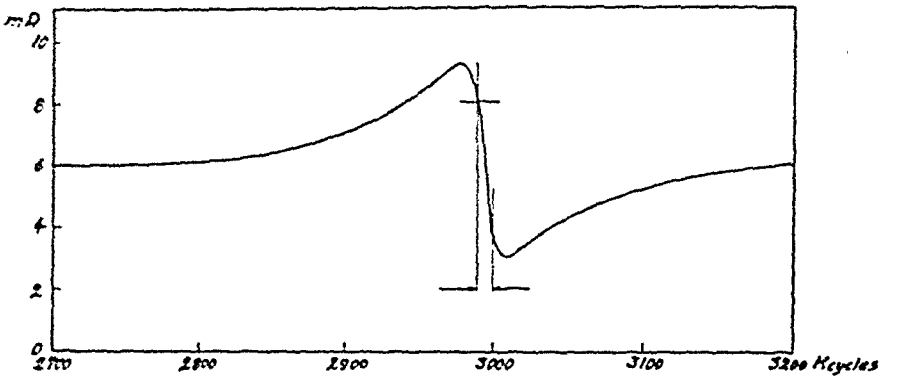

Fig. 6. Variation of anode current as function of frequency in oscillator.
Ordinate: mA.
Abscissa: frequency per sec.

of which was devised by Zakarias (1938) for use in condensor microphones.

In an octode all grids except (4) are used normally i. e. (1) and (2) as oscillator, (3) and (5) as screen grids and (6) as suppressor grid. If grid (4) is earthed the electron-current in the tube will vary proportionally with oscillator frequency which is about 3—5 k-cycles. A weak current of oscillator frequency will enter grid (4) due to influence of the electronic current. The phase difference between this current and tension on grid (1) is seen to be such that its influencing effect may be considered equivalent to a negative capacity between grid (1) and grid (4). With the present octode the size of this capacity is 1—5 pF. If an oscillation circuit "Kg" is introduced between grid and earth and tuned to about the same frequency as the oscillator circuit "Ko", a tension of oscillator frequency will arise in grid (4).

The direct plate current is thus dependent on the transponing properties of the octode and also on the size of phase difference between tension on grid (1) and grid (4) (termed Vg_1 and Vg_4), as the anode direct current will contain a link which is

$$\Delta^i a = K\ Vg_1 \cdot Vg_4 \cos \varphi$$

where cos φ is the phase difference between Vg_1 and Vg_4 and K is a constant. As the capacity equalling influence-effects are slight (large impedance), Vg_1 as well as the current in the oscillator circuit Kg, [i. e. current in grid (4)] may be regarded as ap-

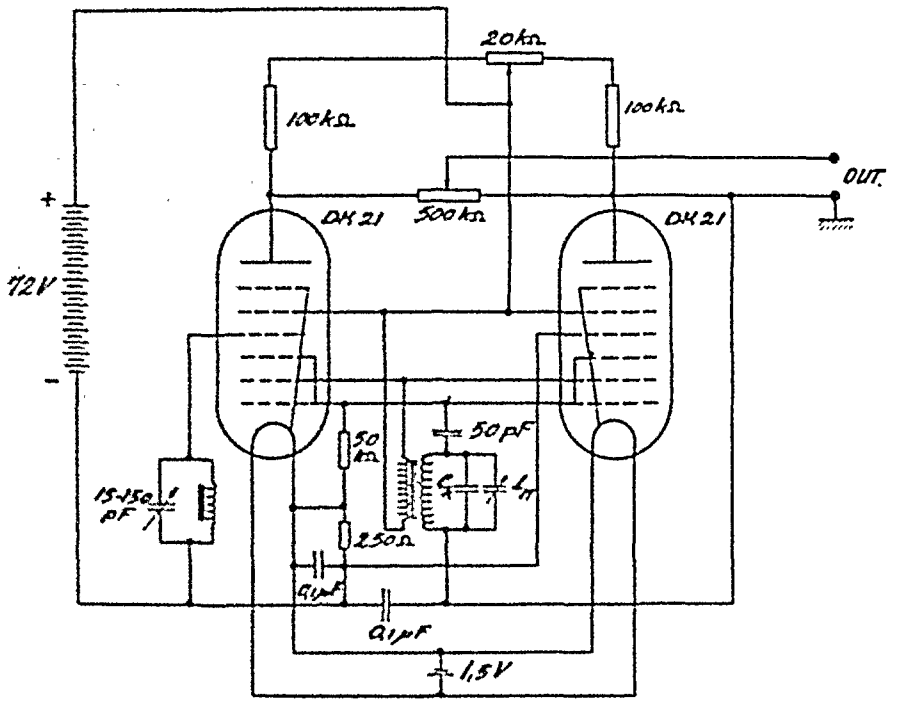

Fig. 7. Balanced high frequency circuit.

proximately constant. If the two circuits are strongly out of phase the impedance of the circuit Kg will be slight ($Vg_4 \cong 0$) and the anode direct current will have its normal value. If the two circuits are only slightly out of phase, Vg_1 and Vg_4 can be reckoned as approximately constant. The changes in the anode direct current Δi_a will be proportional with cos φ.

An exact analysis shows that the anode direct current as a function of tuning frequency for the one circuit when the other is constant, has the course seen in fig. 6. The steep part of the curve is almost linear so that the proportionality between changes of anode direct current (Δi_a) and frequency changes (Δf) is obtained, and when only slighty out-of-tune proportionality is likewise obtained with changes in capacity (Δl). Improvement of linearity and drift properties is obtained by a balanced arrangement (fig. 7) where, with regard to high-frequency, the two octodes work in parallel, with this difference only, that grid (4) on the one octode is directly earthed while that in the other is earthed through the input circuit Kg. By

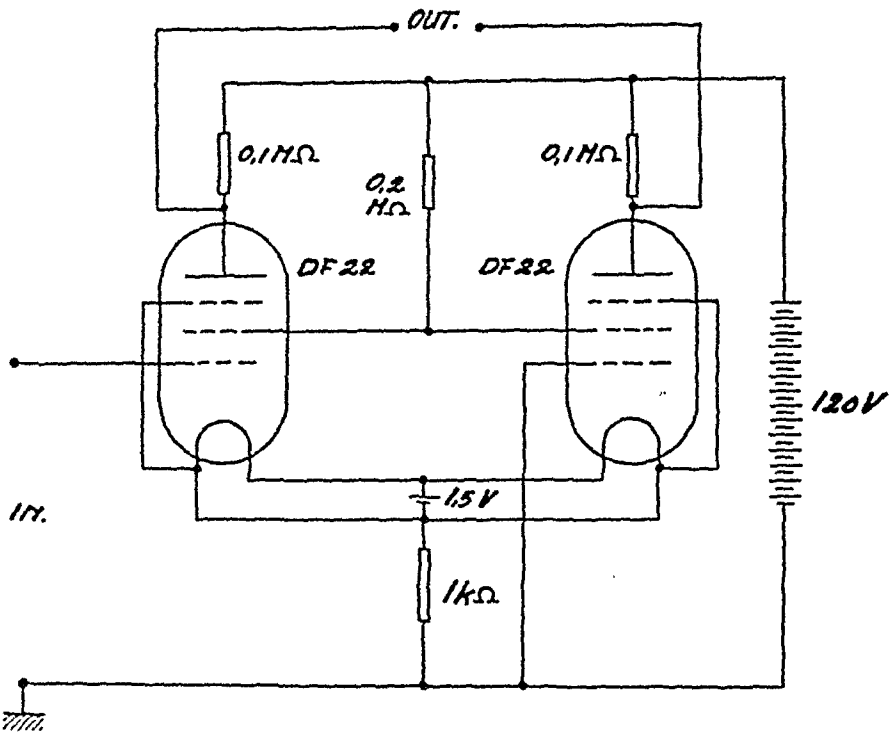

Fig. 8. Balanced direct current amplifier.

placing tension between the two anodes the two tubes will act in the same direction as regards direct tension, whereby an extensive independence of variations in electrode tension and tube properties is obtained. Furthermore the circuits can be arranged so that one plate of the measuring condensor and one output terminal are earthed. Output tension between anodes of the two octodes is amplified in a balanced direct current amplifier (see fig. 8). The balancing which is rather complete due to the common cathode and screen-grid resistances ensures good independence of changes in electrode tension and tube properties.

The movements of the condensor plates are recorded by means of an *electro-static mirror oscillograph* worked by electro-static power only, i. e. it reacts to changes in tension but not to changes in current as do usual mirror-oscillographs.

The vibrating organ of the oscillograph consists of a very thin light metal band (0.0015 mm) which is attached between two pairs

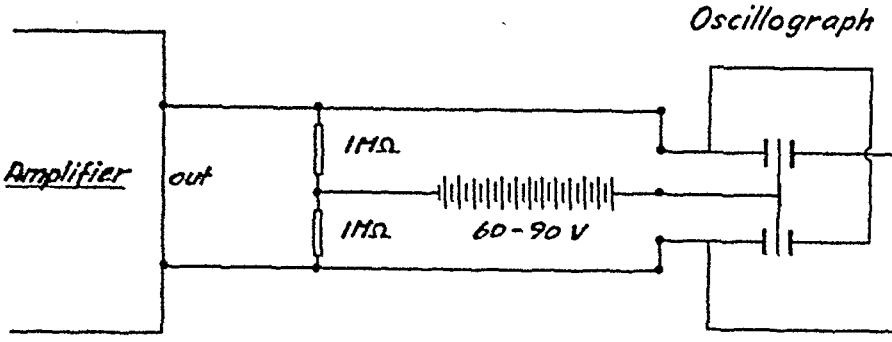

Fig. 9. Coupling of oscillograph to amplifier.

of fixed electrodes at a very small distance from them (0.04 mm). Besides high sensitivity the distance causes mechanical damping of the light metal band. In a somewhat different form the oscillograph is used for sound-film recording by Beer. Under the direction of Dr. F. Buchthal it has been adapted so that it can be used for bio-physical measurements. The technical details of the apparatus are as follows: — resonance frequency 2500 periods per sec; polarisation tension about 90 volt; mirror-surface covered with aluminium; diameter 1.5 mm; lens F = 30 cm. If necessary resonance can be increased to 5000 per sec. but then sensitivity decreases. In these experiments the oscillograph is connected to the amplifier in such a way that the potentials of the electrodes vary while that of the metal band remains constant. The sensitivity of the oscillograph can be altered by changing the polarisation tension by means of a battery (fig. 9).

The *time marker* consists of a rotating spring-driven perforated disc, the speed of which is kept constant by means of a centrifugal regulator. A lamp behind the rotating disc throws light on the film so that time is marked on it at intervals of 1/50 sec. Every 1/10 sec a thicker line is marked.

A camera in which photographic paper is transported at even speed is used for recording. The breadth of the photographic paper can be varied but in all the experiments an unperforated electrocardiographic paper of 6 cm breadth is used. The paper speed can be varied from 1 cm to more than 1 m per sec by a pulley transmission.

The Harvard coil for muscle stimulation is opened and closed at regular intervals by a gramophone motor-driven switch. On the motor axis excentric discs are attached, two of which can close the current circuit for less than a second. The two other discs can close the current circuit for a longer period. These latter are used in stretch experiments where they are arranged so that a certain interval after stimulation the current to the electro-magnet is connected and the muscle stretched and kept so as long as the current is on.

In so-called semi-dynamic experiments frequent stretchings and relaxations are produced by a variable resistance which gives sinus-shaped current variations in the coil of the electro-magnet. Their frequency can be varied partly by pulleys on the motor, partly by varying the revolutions of the gramophone motor. By means of this control vibration-frequency can at most be trebled. In semi-dynamic experiments the frequency is 0.5 and 2.5—4 cycles per sec.

2. Special Method.

Length-tension diagrams.

Length-tension diagrams are investigated at rest and during contraction at room temperature and at 4—6° C. The temperature is kept constant by the cooling device previously described.

The stiffness of the springs is adjusted so high that the distance between the condensor plates (3—4 mm) during contraction, does not change more than 0.02 mm, and thus variations in capacity are practically linear. The change in the distance between the condensor plates corresponds to an equal shortening in the muscle fibre, amounting at most to 0.5 per cent of the total length. This length alteration is slight and contractions can be regarded as isometric.

A *calibration current* sufficiently strong to be easily measured is chosen i. e. one with a deflection of 3—5 mm on the photographic paper.

The length of the muscle is determined by means of a measuring microscope. The vertical line in the eyepiece cobweb is made to coincide with the edge of the micro-tweezer (2) and the microscope tube is moved until it coincides with the other micro-tweezer (11) (see fig. 3). The muscle is stretched by means of a micrometer screw

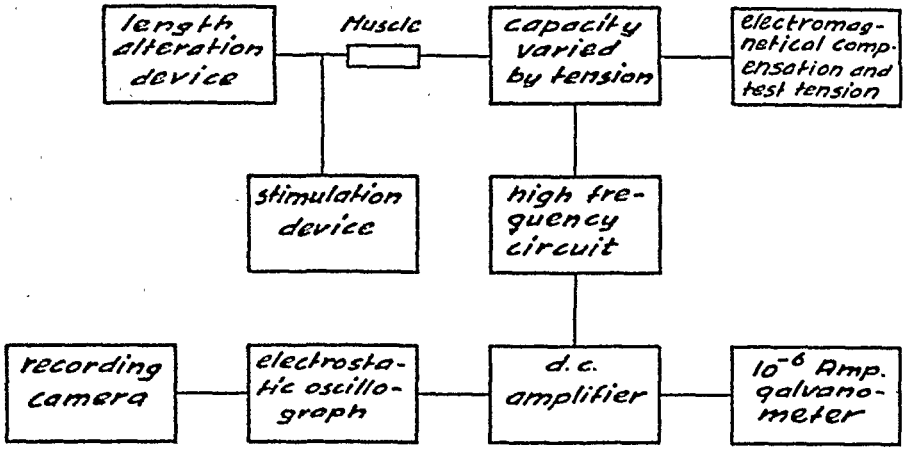

Fig. 10. Block diagram of apparatus for recording tension and stiffness.

attached to the sledge of the fixed tweezer. This stretching is performed by placing the cobweb the distance from the tweezer that the muscle is to be extended and then moving the tweezer with the micrometer screw until it again coincides with the cobweb.

As *original length* we use that where the fibre just begins to develop tension. This is called *equilibrium length* and termed length 100 i. e. 100 per cent stretch corresponds to length 200. It is more difficult to determine for cardiac than for skeletal muscle as the former often has folds which require a certain tension to be straightened out mechanically. Therefore the muscle is stretched 25—30 per cent in advance for a certain period before determining equilibrium length.

At length 100 the muscle is stimulated by opening currents at intervals of 15 sec, by means of the previously described motor-driven switch. Ten contractions are allowed to elapse before recording to ensure a stable base line. After this two calibrating impulses and two to three contractions are registered.

The fibre is then stretched to the next length. The deviation of the movable condensor plate caused by increase in tension is compensated by leading current through the solenoid (8 fig. 3). After sufficient time when the mechanical properties are stabilised i. e. the compensating current remains constant for 3—4 minutes, two contractions are recorded and the compensation current read.

After recording we return to tension zero to ensure that no amplifier drift occurs after the compensating current is removed. If there is a deviation, the amplifier is balanced so that the galvanometer is at zero and the muscle stretched to the length just experimented on, the compensating current read off anew and the last value used. This procedure must be performed rapidly to prevent essential changes in the stretched fibre during control relaxation. The muscle is then stretched to the next length and contractions recorded. At high elongations the muscle takes much longer time to stabilize. At lengths 160—200 consolidation time is about half an hour. As strength of contraction increases with degree of stretch, the sensitivity of the amplifier must be decreased to prevent overloading of the oscillograph. To be able to compensate the constant tension throughout as accurately as at the start of the experiment, the sensitivity of the galvanometer is correspondingly increased.

Deflections due to contractions are controlled on the camera scale so that they are about equal for different elongations.

Stretch and release experiments.

In order to investigate rapid changes in length influencing mechanical properties in cardiac muscle at rest and during contraction, stretch and release length-tension diagrams are registered.

Release and stretch are brought about by the electro-magnet (15) moving tweezer (13). The circuit for current supply of the electromagnet containing rheostats, condensors and a motor-driven switch is seen in fig. 4. Current supply is taken from the mains (130 volt). By inserting suitable condensors stretch and release are not rectangular but rise with a gradient of 0.1—0.2 sec. The recording system, therefore, may be less stiff and more sensitive. By means of a variable rheostat (2000 Ω) the strength of the current and thereby stretch or release amplitudes can be varied. The damping of the tweezer is adapted to the stretch and relaxation velocities and to the stiffness of the myograph.

Sudden Elongations.

Sudden elongations allow an analysis of consolidation (elastic after-effects) at rest and during contraction. The condensor myograph is adjusted to high stiffness and to compensate its low sensitivity,

very small distances between the condensor plates are used (0.2—0.3 mm.).

Changes in the distance of the condensor plates during stretch and contraction of even thick bundles are too small to be visible with the enlargement used in the measuring microscope. The temperature is 4—6° C to provide the longest possible observation time during contraction. At rest, stretch amounts to about 25—30 per cent and is relatively more at low original lengths than at high. The consolidation course is registered for a period of several minutes.

Due to the increase in stiffness the sudden elongations applied *during contraction* must be much less, usually 6—3 per cent of the equilibrium length. The motor-driven switch (fig. 4) initiates with three contacts (1—3), contraction without stretch (1), contractions (2) with stretch (3) at suitable constant level of contraction and constant time intervals. Two to three contractions with and without stretch are recorded for different initial lengths. Also in these experiments care is taken that consolidation has taken place before registration.

Relaxation and Release Experiments.

Decrease in length of the resting fibre is termed relaxation; decrease in length during contraction is termed release. Release and relaxation experiments give information on the reversibility of length-tension diagrams when changes in length are performed rapidly.

In these investigations stiffness of the condensor myograph is decreased. Simultaneously with release experiments, stiffness determinations are performed with the same vibration frequency as that used in other dynamic stiffness measurements, which are described in more detail below (see page 27). When release is performed suddenly, vibrations necessary for determination of dynamic stiffness are distorted by superposed oscillations due to undamped movements of tweezer (1 and 13 fig. 3), and by elastic after-effects. Therefore maximum capacity is introduced in the circuit (fig. 4) to get the slowest release duration (1/5 sec.) The damping of the vibrating system is also somewhat increased in proportion to that used in the previous experiment.

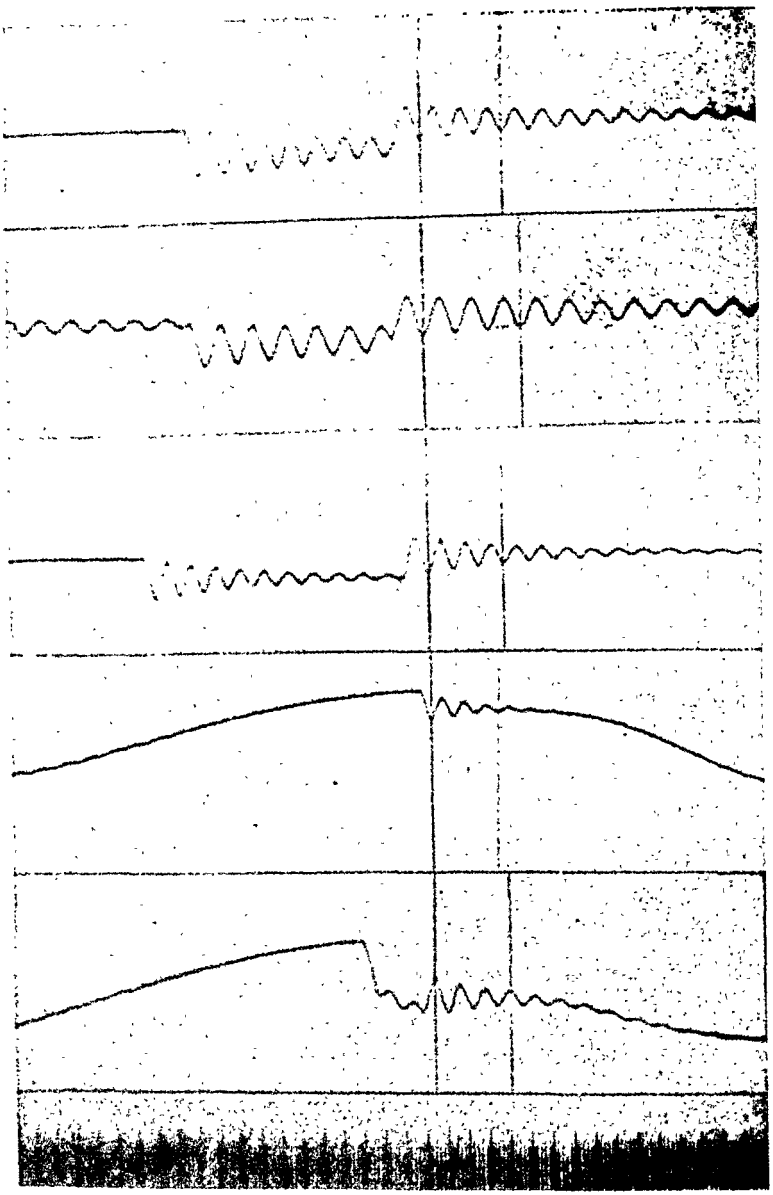

Fig. 11. Determination of dynamic stiffness in vibration experiments.
a. oscillations of the vibrating system without muscle fibre.
b. oscillations of the system loaded with 3 g (determination of the effective mass of the system).
c. oscillations of the system + resting fibre.
d. oscillations of the system + contracted fibre.
e. oscillations of the system + contracted fibre released to the same tension as at rest.
Time marks: = 20 ms.

Release during contraction is adapted so that tension becomes as low as the fibre tension at rest. When relaxing the fibre at rest the changes in length are made similar to those induced in the contracted fibre. Usually two normal and two release contractions are recorded at the same stretch. Here too, recording takes place after previous contractions and when the fibre has been allowed sufficient time for consolidation. Test impulses introduced at rest at the height of normal contractions, and immediately after release in release contractions are used simultaneously to produce vibrations for the determination of dynamic stiffness (see below).

Experiments on dynamic stiffness.

Dynamic stiffness is investigated by means of vibration experiments (fig. 11). The static stiffness can be evaluated from the gradient of the usual length-tension diagram. It is equal to the dynamic stiffness in isotropic bodies e. g. steel, which can be regarded as ideally elastic i. e. consisting of elastic elements only.

A muscle, however, is not an ideal elastic body. It contains, besides elasticity, viscous elements not distributed uniformly over the contractile substance (Levin and Wyman 1927). In vibration experiments the purely elastic properties can be investigated with minor interference from viscosity. By varying frequency all transitions from purely static to purely dynamic properties can be analysed. These vibration experiments thus give better information concerning elastic properties of the muscle than do static length-tension diagrams.

Vibration is brought about by sudden current impulses in the solenoid (8). Stiffness of springs (4 and 5 fig. 3) is adjusted so that vibration frequency lies between 7—12 cycles per sec. At least two contractions with vibrations are recorded at each length preceded by two vibration impulses at rest. The stiffness thus found is the mean of four measurements. Simultaneously vibration impulses allow control of the measuring sensitivity.

The registered curves are measured under a measuring microscope between vibration-maxima and vibration-minima and intervals between corresponding time marks are determined. This control is of im-

Table 1.

1 B	4 B	7 B	10 B
5 v t/800 ms	5 v t/800 ms	5 v t/700 ms	4 v t/600 ms
8,42 8,40 9,52	7,98 8,03 9,32	7,39 7,37 8,11	5,26 5,38 6,51
16,82	16,01	14,76	10,64
T = 0,141	T = 0,137	T = 0,127	T = 0,123
1 K	**4 K**	**7 K**	**10 K**
3 v t/500 ms	3 v t/500 ms	3 v t/400 ms	4 v t/500 ms
4,72 4,76 5,85	4,38 4,34 5,67	4,18 4,14 4,74	5,59 5,54 6,11
9,48	8,72	8,32	11,13
T = 0,134	T = 0,128	T = 0,117	T = 0,114
2 B	**5 B**	**8 B**	**11 B**
4 v t/700 ms	5 v t/800 ms	5 v t/700 ms	5 v t/700 ms
6,08 6,02 7,29	7,76 7,79 9,43	7,36 7,44 8,10	6,93 6,96 8,14
12,10	15,55	14,80	13,89
T = 0,145	T = 0,131	T = 0,128	T = 0,119
2 K	**5 K**	**8 K**	**11 K**
3 v t/500 ms	3 v t/400 ms	3 v t/400 ms	4 v t/500 ms
4,70 4,68 5,75	4,18 4,13 4,57	4,08 4,06 4,52	4,67 4,64 5,16
9,38	8,31	8,14	9,31
T = 0,136	T = 0,121	T = 0,120	T = 0,112
3 B	**6 B**	**9 B**	**12 B**
5 v t/800 ms	4 v t/600 ms	5 v t/700 ms	5 v t/700 ms
7,76 7,79 9,10	6,36 6,31 7,22	7,37 7,28 8,39	6,92 6,91 8,21
15,55	12,67	14,65	13,83
T = 0,137	T = 0,132	T = 0,122	T = 0,118
3 K	**6 K**	**9 K**	**12 K**
3 v t/500 ms	3 v t/400 ms	4 v t/500 ms	4 v t/500 ms
4,59 4,61 5,94	4,40 4,39 4,86	5,45 5,39 6,07	5,31 5,24 5,90
9,20	8,79	10,84	10,55
T = 0,129	T = 0,121	T = 0,112	T = 0,112

T = time in sec. B = resting fibre. K = *contracting fibre.*
v = number of vibrations. t = time in ms.

Table 1.

13 B		14 B		15 B		17 B	
5 v	t/600 ms	5 v	t/600 ms	5 v	t/800 ms	5 v	t/900 ms
5,94	6,90	6,09	6,94	8,95	9,38	10,36	10,92
5,93		6,05		8,95		10,38	
11,87		12,14		17,90		20,74	
T = 0,104		T = 0,105		T = 0,154		T = 0,171	
13 K		**14 K**		**16 B**		**18 B**	
5 v	t/600 ms	5 v	t/600 ms	5 v	t/800 ms	5 v	t/900 ms
5,67	6,64	6,19	7,18	9,00	9,45	10,33	10,90
5,67		6,15		9,04		10,38	
11,34		12,34		18,04		20,71	
T = 0,102		T = 0,103		T = 0,154		T = 0,171	

T = time in sec. B = resting fibre. K = contracting fibre.
v = number of vidrations. t = time in ms.

portance as the speed of paper transport may vary. In Table 1 a record of values for vibration period and time marks is given as an example. From the values found vibration time during different experimental phases is calculated. When vibration time and mass of system are known stiffness can be calculated.

The following formula is valid for an elastic body:

$$T = 2\pi \sqrt{\frac{m}{S}} \quad \ldots\ldots\ldots\ldots\ldots \quad (1)$$

when T = time in sec, m = mass in gram and S = stiffness in dyne cm^{-1}.

Besides the oscillating periods for the recording system + muscle (T_1), that for the unloaded system without muscle (T_0) is determined as well as the oscillating period (T_0) for the system loaded with a known mass M_0 (T_0').

From these determinations of T_1, T_0 and T_0', and formula (1) the following equations can be written

$$T = 2\pi \sqrt{\frac{m_0}{S_0}} \quad \ldots\ldots\ldots\ldots\ldots \quad (2)$$

$$T_0' = 2\pi \sqrt{\frac{m_0 + m_0'}{S_0}} \quad \ldots\ldots\ldots \quad (3)$$

$$T_1 = 2\pi \sqrt{\frac{m_0}{S_0 + S_1}} \quad \ldots\ldots\ldots\ldots \quad (4)$$

From equations (2) and (3) after quadration we get the following expression for the mass of the system (M_0).

$$\frac{T_0'^2}{T_0^2} = \frac{m_0 + m_0'}{m_0} \quad \ldots\ldots\ldots\ldots\ldots \quad (5)$$

$$\because m_0 = m_0' \cdot \frac{T_0^2}{T_0'^2 - T_0^2} \quad \ldots\ldots\ldots \quad (6)$$

After quadration we get from (2)

$$S_0 = 4\,\pi^2 \cdot \frac{m_0}{T_0^2} \quad \ldots\ldots\ldots\ldots\ldots \quad (7)$$

Substituting (6) for (7)

$$S_0 = 4\,\pi^2\,\frac{m_0'}{T_0' - T_0^2} \quad \ldots\ldots\ldots\ldots \quad (8)$$

By quadrating (4) we get the following expression for stiffness of the fibre (S)

$$S_0 + S_1 = 4\,\pi^2\,\frac{m_0}{T_1^2}$$

$$\because S_1 = 4\,\pi^2\,\frac{m_0}{T_1^2} - S_0 \ldots\ldots\ldots \quad (9)$$

If, besides stiffness of the fibre, its length (l) and cross-section (q) are known, the elasticity modulus (E) can be computed. We get

$$E = \frac{S_1 \times l}{q} \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (10)$$

In the majority of cases q is not determined and only relative values of stiffness are used to characterise elastic properties. The relative elasticity modulus computed in a number of cases is derived from formula

$$E_m = \frac{S_m}{S_{100}} \cdot \left(\frac{l_m}{100}\right)^2 \quad \ldots\ldots\ldots\ldots \quad (11)$$

where S_m is stiffness measured at length l_m, S_{100}, equilibrium length and E_m, the relative elasticity modulus.

The current impulses inducing vibrations must not be so strong that they cause appreciable length alterations of the muscle, as considerable changes in tension would result. They are therefore adapted so that the changes in length are not more than half those caused by the isometric contraction i. e. at most 0.25 per cent of the length of the muscle.

To estimate stiffness as accurately at possible it should be similar in both muscle and myograph i. e. it must lie between maximal and

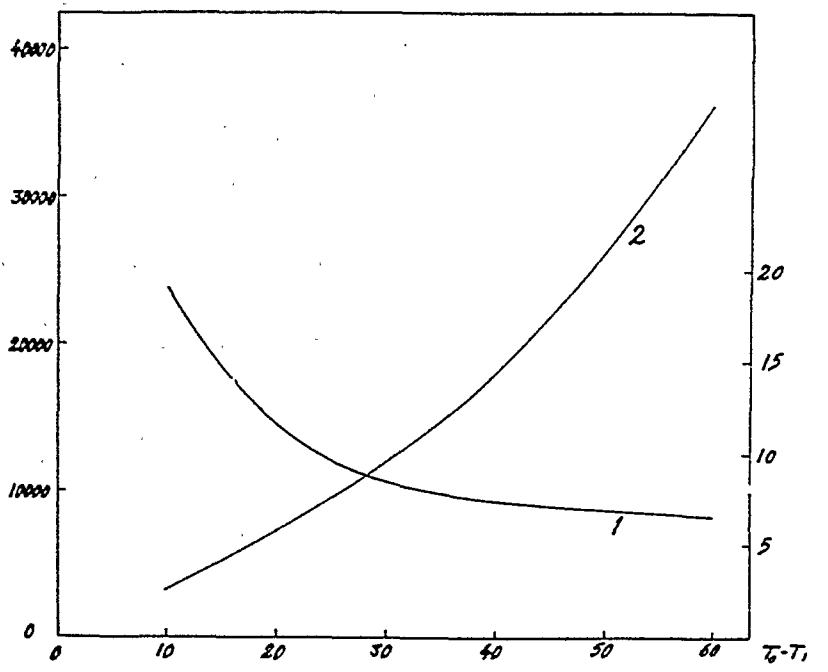

Fig. 12. Curve 1. Error in determination of stiffness (measurement error 2 ms) as a function of oscillation period ($T°$—T_1).
Curve 2. Stiffness as function of variation in oscillation period.
Abscissa: variation of oscillation period in ms.
Ordinate: (to the right belonging to curve 1) percentage error. (to the left belonging to curve 2) stiffness in dynes cm^{-1}.

minimal stiffness of the muscle. Stiffness and the mass of the system are adjusted to produce frequencies which ensure determination of dynamic stiffness proper even at the lowest frequencies. If this is not the case the oscillating period affects stiffness which becomes relatively higher at high than at low frequencies.

With a frequency of 7—13 cycles per sec, the mass of the system is 10—12 g. This frequency allows stiffness at the height of contraction to be calculated from up to 7—8 vibrations which all lie within the peak level of contraction where tension is relatively constant. It is also possible to measure stiffness in release experiments during contraction but here maximally 4—5 vibrations fall within the same level of contraction. This naturally holds good only for experiments at low temperature.

To determine accuracy in measuring dynamic stiffness, experiments are performed on well consolidated fibres at different stretch. Four determinations are made at each length. The maximal error does not exceed 2 ms with T varying between 145—100 ms. The curves in fig. 12 show how this error varies as function of the difference in T_0—T_1.

Simultaneously with experiments on dynamic stiffness, length-tension diagrams are recorded and from their gradient static relative E-moduli of the resting fibre are calculated and compared with dynamic. To get an idea of the absolute size of these moduli the *fibre diameter* is measured in two planes at right angles to each other, presupposing that the edges of the preparation are rounded so that the shape of the cross-section would be something between a rectangle and an ellipse. Although this procedure is imbued with relatively high uncertainty it is preferable to weighing because the preparation weighs less than 1/10 mg, and uncontrollable evaporation of its fluid would introduce still greater errors. The diameters are measured by placing a surface-plated mirror in the preparation chamber (23 fig. 3) so that it reflects at an angle of 45° and thus makes possible a measurement of the fibre dimensions in two planes at right angles to each other. Fibre diameters and their reflected images are measured with the eyepiece micrometer of the measuring microscope. The E-modulus can thus be calculated from formula 10 and the data found above.

Semi-dynamic experiments.

The above vibration experiments are well suited to an investigation of stiffness within a small range but less so for a continuous study of stiffness. For this the fibre must be subjected to periodic changes in length which cause corresponding changes in tension (Buchthal, Kaiser and Knappeis 1944). These are an expression of the fibre stiffness which can, therefore, be calculated if changes in length and corresponding changes in tension are known.

The changes in length in the fibre are brought about by means of the electro-magnet (15 fig. 3) and of a resistance, the size of which can be varied by a motor-driven slider thereby producing the sinus-

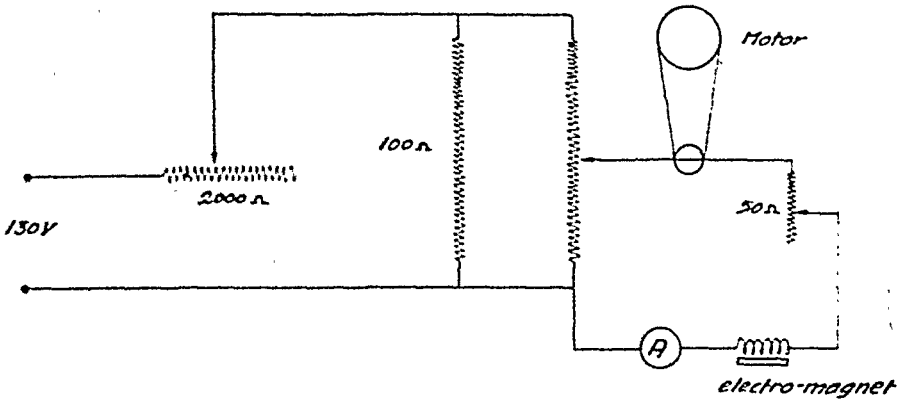

Fig. 13. Diagram of electro-magnet and variable resistance.

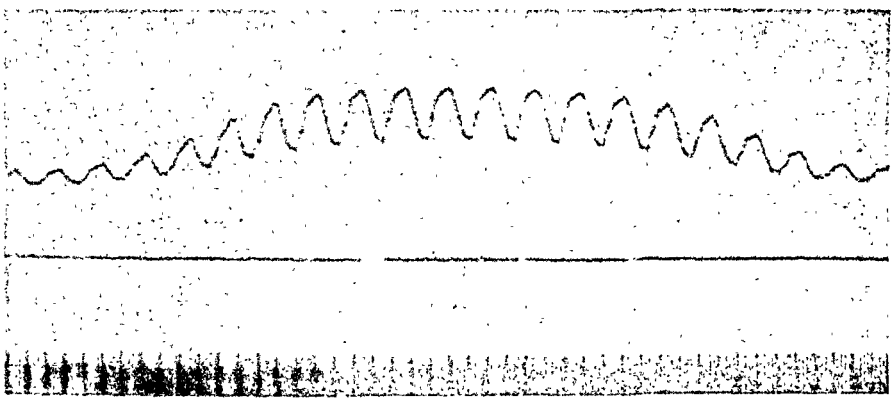

Fig. 14. Determination of stiffness from periodic length-tension variations. Time marks = 20 ms.

shaped current variations described above. A scheme of the arrangement is given in fig. 13. Changes in tension are recorded by the condensor myograph. Changes in length can amount to between 6—8 per cent of the fibre length. Frequency used is between 2—4 vibrations per sec.

Thus during contraction the tension developed is superposed with small tension variations which increase with increasing contraction tension and their size is a measure of stiffness (fig. 14). A measurement of tension is given by the mean of the upper and lower curves.

To enable the oscillating condensor plates to follow these changes in tension the stiffness of the hanging springs must be considerably higher than in the dynamic experiment. The springs are therefore adjusted to a vibration frequency of about 100/sec. The distance between the condensor plates must be decreased in a corresponding degree to obtain sufficient sensitivity. After recording calibration impulses, periodic tension variations are introduced and 2 contractions recorded. Otherwise the experimental procedure corresponds to that described in the dynamic experiments.

The curve is analysed in the following manner. A base line is drawn parallel to the edge of the film. Then the peaks and dips in the sinus-formed vibration curves as well as the base line are perforated by a fine needle. The curve is then placed in a projection apparatus which gives a sufficient enlargement (about 3—4 times) and the light dots corresponding to the perforations are drawn on millimetre paper. The points of the peaks and the dips are joined to make an upper and lower curve and the base line drawn. The differences between the distance from the base line to the upper and lower curve which corresponds to stiffness are read for every 5 mm. The mean between the sums of the distances, which is a relative measurement of the tension present, is calculated. The height of the projected calibration impulses is read and from these data values of stiffness and tension are computed.

Investigation of viscous properties.

Decrement of amplitude in vibrations in the stiffness experiment described above gives a measure of viscosity. From these the so-called damping constant can be worked out. This is a measure of the energy inhibiting the above vibrations. Energy comes from the muscle itself as an expression of its viscosity. The proportion between two succesive vibrations can be expressed by the formula.

$$f = e^{P\,T/2\,m.} \quad \ldots\ldots\ldots\ldots\ldots \quad (1)$$

where P = damping constant, T = time in sec and m = mass of the vibrating system in gram.

P is expressed in dyne $\times$ cm^{-1} $\times$ sec and is an expression of the non-elastic resistance in dynes inhibiting the system when its rate of movement is 1 cm/sec.

From equation (1) we get

$$P = \log f \frac{2m}{T} \quad \ldots\ldots\ldots\ldots\ldots \quad (2)$$

P is thus affected by the ratio between the amplitudes of two successive vibrations and there is a direct proportion to the natural logarithm of these ratios and an inverse proportion to vibration time.

By measuring the amplitudes and plotting them in a semi-logarithmic coordinate system with abscissa marking frequencies with constant distance, a gradient of the curves proportional to the logarithmic decrement is obtained. If we call the difference between the two amplitudes on these curves L, and the decrement in a line corresponding to the degree of damping h_e, equation (2) can be written

$$P = \frac{h}{h_e} \cdot \frac{2m}{T} \quad \ldots\ldots\ldots\ldots\ldots \quad (3)$$

This is an expression for total damping. To find the damping of the muscle P_f, the damping of the system itself P_a must be subtracted.

$$\text{We get } P - P_a = P_f \quad \ldots\ldots\ldots \quad (4)$$

For a better understanding of the size of the viscous forces compared with the elastic stiffness of the fibre, the so-called viscous stiffness is worked out from the formula.

$$St_v = P_f \cdot 2\pi \frac{1}{T\,(\text{sec})} \quad \ldots\ldots\ldots\ldots \quad (5)$$

Curves from the dynamic experiments are used for this viscosity calculation. They are treated in the same way as those for stiffness determination and suitable amplitudes of oscillations are chosen to allow convenient measurement.

The peaks and the minima respectively are connected, the distance between the upper and lower curve determined at each peak and each minimum and plotted in a semi-logarithmic coordinate system. The logarithmic decrement is then read and enters the above formula for calculating damping constant and viscous stiffness.

Consolidation course following sudden elongations of the fibre at rest and during contraction — the so-called elastic after-effect — just as the damping constant, is a measure of viscosity. Apart

from these experiments with short observation time, a series of experiments is performed where consolidation time is followed for several hours.

The apparatus is the same as that used by Buchthal, Kaiser and Knappeis (1944) in similar experiments on skeletal muscle. It is constructed for measuring consolidation of the muscle at different degrees of stretch and temperature, and consists of two tweezers, one fixed to a slide moved by a micrometer screw, the other suspended in a gallows by two thin steel springs 9 cm long. To avoid conduction of heat between tweezer and springs, the former is attached to the springs by short pieces of thin wire (0.1 mm diameter) of low thermal conductance. The tweezers are made of Invar metal to prevent thermal expansion affecting the experiment. Displacements of the springs and tweezer are microscopically observed by measuring movements of a cobweb attached to the latter. The enlargement amounts to about 50 times and cobweb displacements are measured by means of an eyepiece screw micrometer with movable cobweb (1 scale unit = $2 \cdot 4$ μ). Preparation, Ringer solution and cooling are the same as in previous experiments. The fibre is stretched 20—30 per cent for ½ hour. Then equilibrium length is determined and the fibre stretched anew 25—35 per cent. Tension changes are then read by the eyepiece micrometer every 10—15 seconds. After a few minutes, intervals between readings are suitably increased. After two hours or sooner if consolidation finishes before, the new equilibrium length and fibre tension are determined by relaxation to tension zero. Consolidation course is then followed again after a new elongation of 25—30 per cent, for a period of up to 3½ hours.

Apart from experiments with sudden stretches, those with sudden relaxations can provide information on plastic and viscous properties of the fibre.

Investigation of the lengths of isotropic (I) and anisotropic (A) substances.

A number of experiments are performed to measure the part in height of compartment taken by the anisotropic and isotropic substances respectively. Single fibres at the edge of a thin bundle of cardiac muscle fibres are micro-photographed at rest and during

contraction and the negative measured microscopically (for measuring technique c. f. Buchthal, Knappeis and Lindhard 1936). Thin muscle bundles of cardiac muscle (6—8 fibres) from the wall of the ventricle are fixed at both ends under two metal clamps fastened to two metal blocks which slide in a sledge in an ebony plate. The preparation can be stretched or relaxed by a micrometer screw.

Micro-photographs were taken with microscope objective Zeiss epi. 40 numerical aperture 0.65 or apo. 70 numerical aperture 1·125. The enlargement amounted to 200—350 times. The highly sensitive film, Ilford Pan hypersensitive was used as negative material which in conjunction with a Wolfram point light lamp (arc lamp) allowed an exposure of 0.1—0.2 sec which is sufficiently short to photograph the cooled preparation during contraction.

Measuring of negatives.

Micro-photographs are measured on the negative under the microscope with an eyepiece screw micrometer with adjustable cobweb enlarging 12·5 times. Total enlargement thus amounts to about 2500—4200 times respectively. To obtain the height of compartment the length of 10 successive compartments is measured. Besides this, 10 measurements of I and A substance respectively are taken on each negative.

Experiments with application of adrenalin and acetylcholine.

Preliminary experiments are performed on the effect of adrenalin and acetylcholine on mechanical properties of cardiac muscle (tension, stiffness and viscosity) during rest and contraction.

Adrenalini hydrochlor. Leo in ampulla 1.2 mg/ml is used. Before use the solution is diluted with frog Ringer to a concentration of 1:200.000. The acetylcholine solution is prepared from crystalline substance (Hoffmann la Roche). Acid solution with a pH of 5 obtained by adding 0.1 n HCl is found to be stable (Brown, Dale and Feldberg 1936). During the experiment adrenalin and acetylcholine are added in concentrations previously found to affect

muscle tension and causing at least 20 per cent increase or decrease respectively. Concentration lies between $1:5\cdot10^6$ and $1:10^7$. Length-tension diagrams during rest and contraction as well as length-stiffness and stiffness-tension diagrams in both vibration and semi-dynamic experiments are investigated. Furthermore damping is determined in vibration experiments. All other experimental conditions are the same as described above and fibre lengths from 100—125 are investigated.

An experiment was performed in the following way. Some calibrating impulses were recorded with 2—3 normal contractions. Adrenalin or acetylcholine was added to the Ringer solution by a fine syringe, care being taken to ensure that the drug was well distributed. After 3—4 minutes conditions were stabilised. Then the compensation current was read at lengths over 100 and calibrating impulses and contractions registered as above. The preparation was then washed out with Ringer solution and control contractions recorded. Generally normal conditions were restored 3—4 minutes after the washing out. The muscle was then relaxed and the apparatus controlled for possible drift. Then the muscle was stretched to the next length and the same procedure repeated.

Results

Length-tension diagrams of cardiac muscle.

In previous investigations of mechanical properties of cardiac muscle the static properties (consolidated tension as function of stretch) have been the especial object of study. As pointed out by Gasser and Hill (1924) and Buchthal (1942), these experiments are only an incomplete expression of elastic and viscous properties. They provide information on static stiffness, plasticity and work capacity. Besides purely static experiments the following chapters deal with so-called dynamic and semi-dynamic experiments including stretch and release of the resting and contracting muscle. Here the simultaneous variation of stretch and tension are recorded with frequencies of 10 and 1—0.5 cycles per sec respectively. The changes in length of A and I substance during stretch at rest and during contraction are investigated and the length-tension diagrams for the respective substances constructed.

The length-tension diagram at rest (fig. 15 a) rises gradually in the beginning and more steeply from length 170—190. Stretches up to 200 per cent of equilibrium length are attained here. The first part of the curve up to length 120 shows a tendency to rise more steeply. Otherwise the curve is evenly concave upwards. The first abrupt rise in the length-tension diagram of the resting fibre, found also in rubber, does not occur in skeletal muscle. This implies that at equilibrium length cardiac muscle is less pre orientated than skeletal muscle and may explain the difference in extensibility. Determination of equilibrium length leads to more constant and exact values when the muscle is previously stretched 30 per cent. Equilibrium lengths with and without short pre-stretching may differ up to 10 per cent.

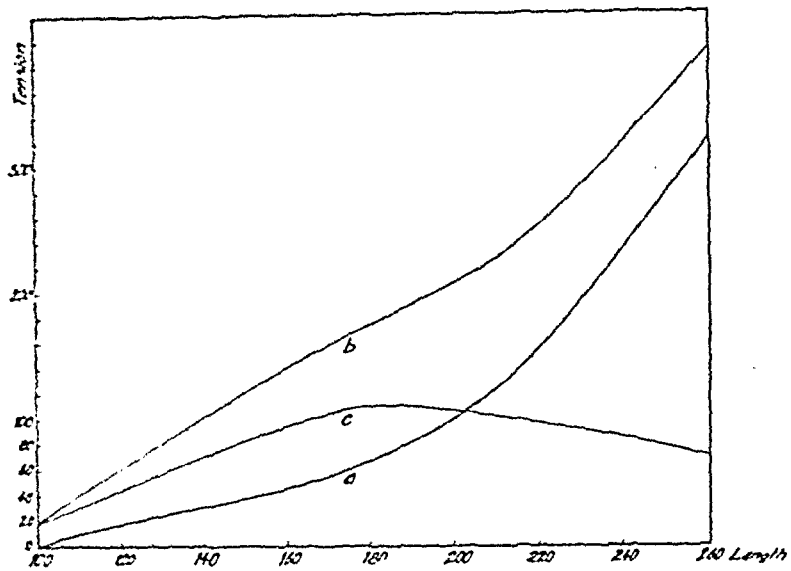

Fig. 15. Length-tension diagram of a cardiac muscle bundle at rest and during isometric contraction.

a. length-tension diagram at rest.

b. length-tension diagram during contraction (*initial tension* + extra-tension = isometric maxima).

c. extra-tension produced by contraction.

When extension exceeds 25 per cent it is not completely reversible in the minute during which it is observed. When a fibre is extended to length 200 in the course of an experiment of long duration, and then released to tension zero, a lengthening of more than 60 per cent persists, provided that not more than a minute has elapsed between relaxation and measurement. After a few more minutes a further shortening takes place. This slow stabilisation of the fibre can be explained by viscosity (c. f. page 67).

Length-tension diagram during isometric contraction (fig. 15 b). Up to length 175—200 the curve of the contracted fibre (isometric maxima) rises more steeply than when at rest. At higher elongations the contraction curve approaches the length-tension diagram of the resting fibre. This convergence is never so great that the curves lie as close to each other as they do at length 100. The shape of the length-tension diagram during contraction is dependent on extra-

tension produced by the contraction which increases up to length 175—200 (fig. 15 c). At higher elongations extra-tension decreases. In many cases however, no decrease in extra-tension of the fibre is attained as during contraction it frequently snaps at lengths 225—250. Extra-tension of contraction can attain, at maximum, values 6—8 times higher than at length 100.

The length-tension diagram of contracted cardiac muscle differs essentially from the corresponding curves for *skeletal muscle* where, often at equilibrium length, extra-tension attains its maximum, then decreases and finally meets the length-tension curve of the resting fibre at length 160—200. The sudden changes in gradient of length-tension diagrams of skeletal muscle during contraction, observed by Blix (1892) and others, and explained by Buchthal (1942) as being due to yielding within the contractile substance, are not found in length-tension diagrams of cardiac muscle.

Stretch of muscle during contraction.

When recording the curve for the isometric maxima the muscle is given different lengths *at rest* and then contractions are initiated. With stretch *during contraction* (3—4 per cent) increase in tension measured 1 sec after stretch rises more steeply than it does in the curve for the isometric maxima (1. fig. 16.). The higher the initial length the steeper is the increase in tension. These observations are in contrast to similar experiments on single skeletal muscle fibres (Buchthal 1942) where curves for isometric maxima and length-tension diagrams for the muscle stretched during contraction coincide up to length 140. At higher elongations, tension after stretch during contraction of cardiac muscle likewise exceeds tension values of isometric maxima.

Release of contracting muscle to tension at rest.

The fall in tension in release contractions shows a course which is very similar to length-tension diagrams obtained in stretch experiments i. e. tension attained during release contraction lies considerably below the isometric tension at the same length. The fall in tension does not follow that for isometric maxima which apparently is not reversible. Here also tension changes at higher initial

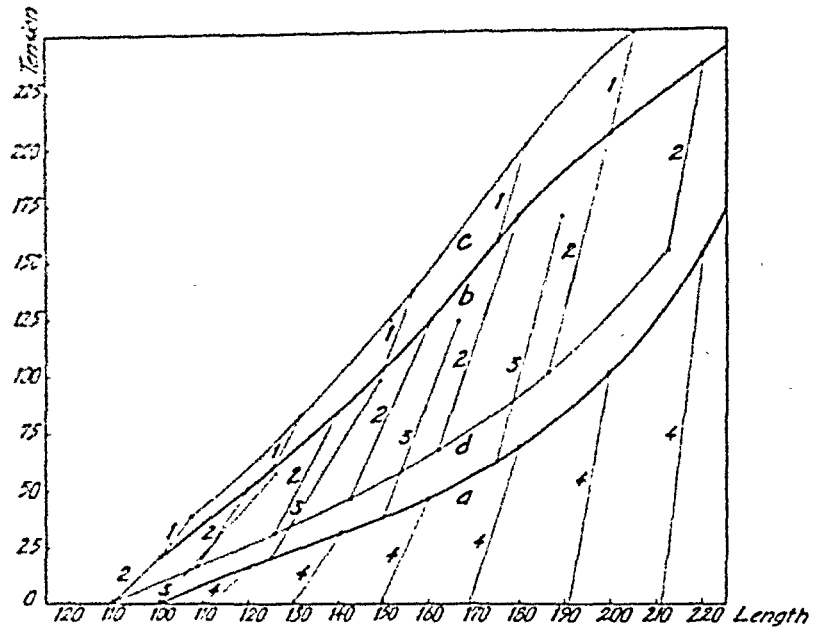

Fig. 16. Length-tension diagram of a cardiac muscle bundle at rest and during contraction (mean curve).
a. length-tension diagram of resting fibre.
b. length-tension diagram of isometrically contracting fibre.
c. length-tension diagram of muscle stretched during contraction.
d. length-tension diagram of muscle released during contraction.
(1) variations in tension when stretching from isometric maxima.
(2) variations in tension when releasing from isometric maxima.
(3) variations in tension when stretching a resting fibre.
(4) variations in tension when relaxing a resting fibre.

lengths are steeper (2. fig. 16). Changes in length during these release contractions are at most 30 per cent of the equilibrium length.

The course of release curves is like those found in skeletal muscle during release from tetanic contraction, where a consolidation time of five seconds is allowed, which is sufficient for the fibre to consolidate. In spite of the possibility of consolidation in skeletal muscle the partial release length-tension diagram does not coincide with the curve for the isometric maxima. This deviation is explained by introducing the conception of an *elastic locking* (Buchthal 1942) irreversible during contraction. The release diagrams represent a system of approximately parallel curves starting from the respective points of the curve for the isometric

maxima. The fibre is regarded as being locked to one of these partial diagrams as long as stimulation continues, and the fibre is not stretched above the respective lengths in isometric contraction.

The question now is whether these release curves in cardiac muscle are likewise an expression of elastic locking or are due to incomplete consolidation. With regard to static conditions it is obvious that in contracting cardiac muscle, tension can only be observed during a much shorter time than in corresponding experiments on skeletal muscle. Part of the great fall in tension measured between the isometric maxima and release curve must therefore be due to the essentially shorter consolidation time. How large a part of the fall in tension is actually static cannot be deduced from experiments during contraction, but a corresponding relaxation at rest can supply this information.

Relaxation of resting muscle.

Tension here is registered at the same time after relaxation as tension during release contraction (4 fig. 16). The length-tension curves during relaxation show a course completely parallel with release curves during contraction. *The fall in tension is thus due to incomplete consolidation time and is not static.* In skeletal muscle a great difference is found between relaxation at rest and release during contraction, which cannot be explained by viscosity as can the corresponding fall in tension in cardiac muscle and which is due to elastic locking.

Correlation of length-tension and intraventricular pressure.

The curve of the isometric maxima in cardiac muscle differs from the corresponding curve for skeletal muscle in that it shows a steeper rise throughout so that contraction extra-tension only reaches its maximum at much higher elongations. Apparently this observation disagrees with that of O. Frank (1895) on the whole ventricle. He finds that pressure during contraction has already reached a maximum at moderate degrees of filling.

What is the explanation of this difference between results found for the whole ventricle and for the muscle bundle? There are four main possibilities. *Firstly*, the increase in tension in muscle due to an increase in volume may not result in an increase in pressure, as the resulting pressure, though proportional to the increase in tension,

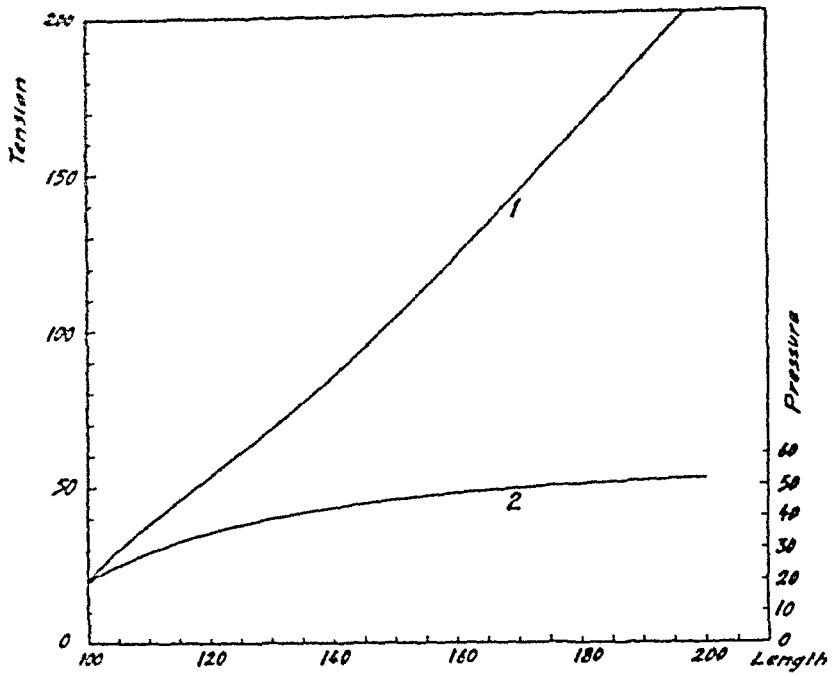

Fig. 17. Relation between tension in cardiac muscle and intraventricular pressure.
(1) length-tension diagram.
(2) length-pressure diagram.
Abscissa: length of fibre (equilibrium length = 100).
Ordinate: tension and pressure in identical arbitrary units.

is in inverse proportion to the second power of the radii, if we suppose tension concentrated in the middle of the wall. This inverse proportionality between pressure and radii cannot, however, explain the difference between Frank's results and ours. Fig. 17 shows the relation of tension (isometric maxima) and systolic pressure as function of length of the single muscle element. The heart is presumed to be spherical. Tension increases more abruptly than pressure. At length 200 pressure is only quarter as high as tension while volume is 8 times greater than initial volume i. e. the heart volume with all its fibres at equilibrium length. The curve in fig. 17 deviates from Frank's curve in that the pressure corresponding to tension in the curve for isometric contraction does not attain a maximum. Therefore, the diminution of relative tension with increasing radius cannot explain the difference between Frank's observations and ours.

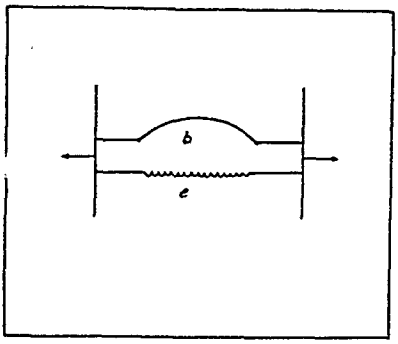

Fig. 18. Diagram of elastic and non-elastic resistance to stretch.
e = elastic forces consisting of contractile elements.
b = non-elastic, non-contractile elements.

The *second possibility* is that experiments on cardiac muscle fibres only correspond to the lower part of Frank's curve. This alternative is hardly probable as the changes in length present in our experiments would conform to 10 times the initial volume of the heart, while Frank, in experiments on the whole ventricle, finds only lesser changes in volume.

Thirdly, it is possible that a great deal of tension in the resting fibre in Frank's experiments is caused by shunting effect of relatively non-elastic elements such as connective tissue, vessels and pericardium. How this shunt effect works is seen in fig. 18 where e is an elasticity which when stretched develops force e, and b corresponds to several non-elastic elements developing force b. During contraction we get an extra tension f, which does not appear fully but becomes f—b, as b disappears with shortening provided that it is sufficiently non-elastic.

The *fourth possibility* is that viscosities arranged parallel in the heart added to those of single fibres can have an inhibitory effect on tension development.

Shunt effect and viscosity, possibilities three and four, are the most probable explanations of the difference between our length-tension diagrams and Frank's pressure-volume curves.

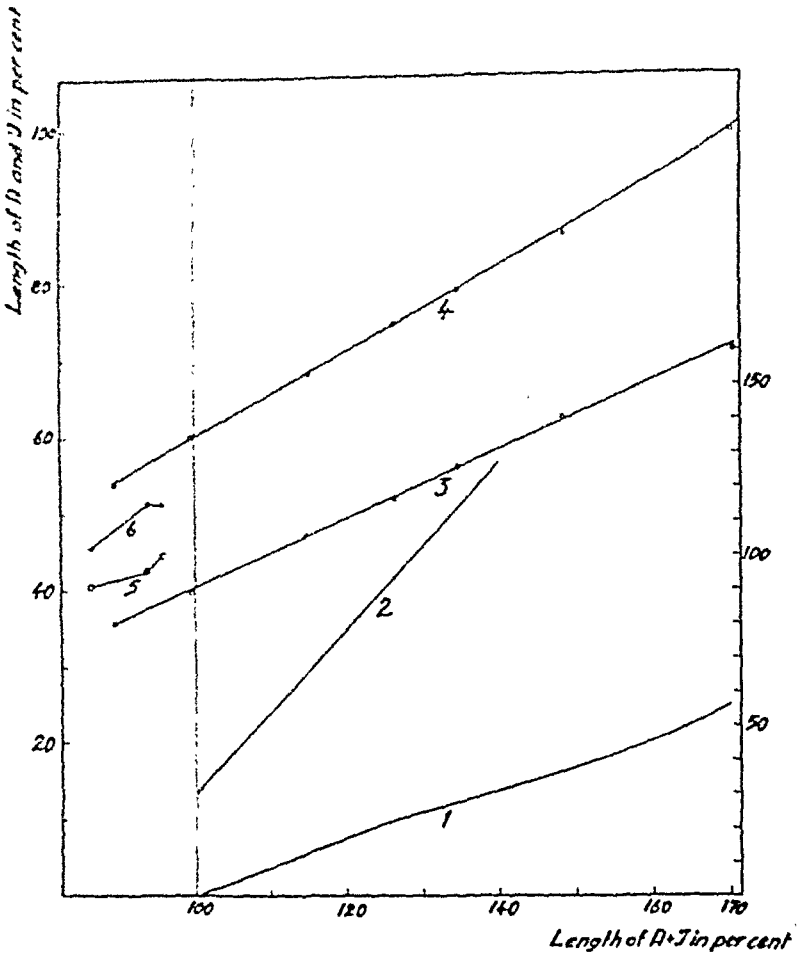

Fig. 19. Length of anisotropic (A) and isotropic (I) substances in per cent of equilibrium length of compartment (A + I) at rest, compared with the length-tension diagram of the fibre.

(1) length-tension diagram of the resting fibre.
(2) curve of isometric maxima (first part).
(3) length of I at rest as function of stretch.
(4) length of A at rest as function of stretch.
(5) length of I during isometric contraction.
(6) length of A during isometric contraction.

Abscissa: (common for length-tension diagram and for the relative lengths of A and I) height of compartment (A + I).

Ordinate: *(left)* height of A and I in per cent of A + I at equilibrium length, *(right)* tension of cardiac muscle fibre in relative units.

Length-tension diagrams of the anisotropic and isotropic substance*).

The proportion between the length of the anisotropic (A) and isotropic (I) substance at different degrees of stretch is studied in resting cardiac muscle in 24 experiments. The length of the fibre varies between 100 and 170. During contraction only a few preliminary experiments are performed at equilibrium length. In these experiments only the lengths of A and I substance as a function of stretch are studied, as for technical reasons stiffness and tension cannot be investigated simultaneously. Using mean curves of length-tension diagrams from other experiments, the respective diagrams for the A and I substances can be constructed.

The length of both A and I at rest as a function of stretch shows a linear increase (fig. 19) which is relatively greater for I than for A. At equilibrium length the A substance is 60 per cent of the length of compartment and the I substance 40 per cent, while at higher elongations they are 58.8 and 41.2 per cent respectively. This difference is more marked if the relative increase in length of the substances is referred to the respective equilibrium lengths (fig. 20). The relative increase in length for I is essentially higher than that for the A substance, e. g. at length 110 the I substance has increased by 12 per cent while the A substance has only increased by 8 · 5 cent. The A substance is thus 30 per cent less extensible than the I substance. This is in contrast to skeletal muscle where I is 20 per cent less extensible than A.

The length-tension diagram of the A and I substance at rest.

Length-tension diagrams for the respective substances can be constructed from the curve of the length of A and I as function of stretch (3, 4 fig. 19) and the length-tension diagram of the resting fibre (1. fig. 19). We suppose that all tension in the muscle fibre is exerted by the A and I substance and disregard other factors which could affect elastic properties e. g. sarcolemma and other membranes inside the muscle fibre, the contribution to tension of which

*) These experiments were performed in collaboration with Mr G. G. Knappeis.

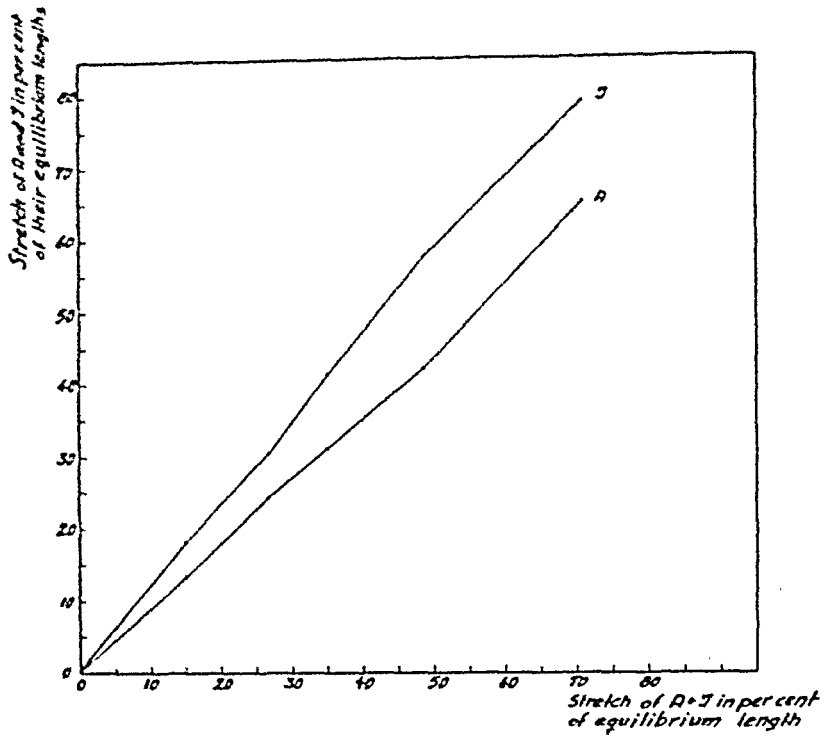

Fig. 20. Stretch of A and I in per cent of their equilibrium lengths as a function of stretch of compartment (A + I).
Abscissa: Stretch of compartment (A + I) in per cent of equilibrium length.
Ordinate: Stretch of A and I in per cent of their equilibrium lengths.

is not supposed to exceed a few per cent. The length-tension diagram for the I substance has a steeper course than that for the A substance, otherwise the curves have a similar course (fig. 21).

As preliminary *contraction experiments* were hitherto performed only at equilibrium length, comparisons with length-tension diagrams at rest could not be drawn. During contraction the A substance shortens about 10 per cent while the I substance extends about 15 per cent (fig. 19). Thus contraction tension appears to emanate more from A than from I. In how far the I substance participates actively in contraction is difficult to determine from these experiments alone. From the length-tension diagram of I at rest it can be seen that an increase in length of 15 per cent i. e. from 40—46 results in only half the tension developed during contraction. This, with

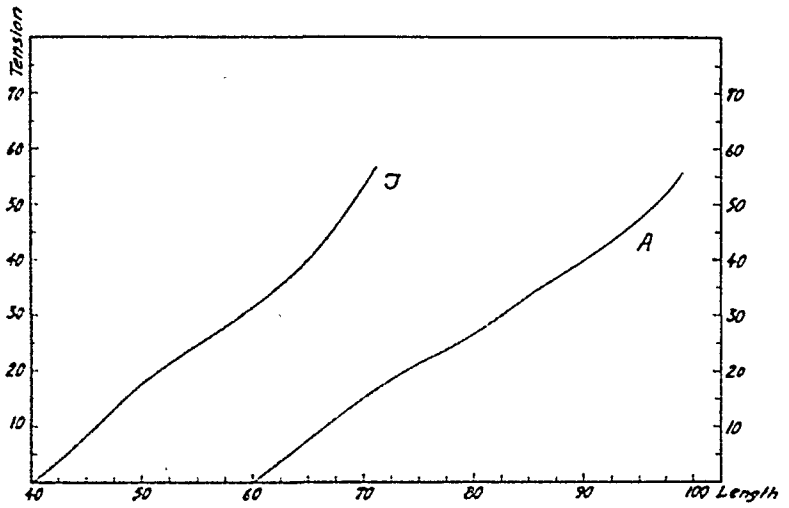

Fig. 21. Length-tension diagram of A and I substances at rest.
Abscissa: length of A and I in relative units (equilibrium length of A+I=100).
Ordinate: tension in relative units.

numerous observations by Buchthal (1942) on skeletal muscle, supports the assumption that also in cardiac muscle the I substance participates actively in the contraction process. The essential proof of the activity in the I substance in skeletal muscle is seen in release contraction experiments which unfortunately, due to reasons discussed above, cannot be performed on cardiac muscle.

Investigation of stiffness.

Stiffness in vibration and semi-dynamic experiments is investigated at rest, during isometric contraction and release contraction as function of length and tension (method page 27). Table 2 contains a record of a vibration experiment. The stiffness values obtained are relative and in a series of experiments absolute elasticity moduli are determined. When determining elasticity modulus, variations in cross-section which are considerable in highly elastic bodies are introduced. Therefore, when investigating *structural properties* in relation to tension in highly elastic substances e. g. muscles, the elasticity moduli as emphasised by Buchthal (1942) are not the best expression for the experimental observations. As stiffness-tension variations are primarily determined from changes in molecules or micellae, and less

Table 2.

Length	Number of registration	Compensation current in mA	Calibrating current in mA	Heigth of calibration in mm	Heigth of contraction in mm	Tension at rest in mg	Extra-tension during contraction in mg	Tension during contraction (Isometric maxima) in mg	Vibration time at rest in sec	Stiffness at rest in dyne cm-1	Vibration time during contraction in sec	Stiffness during contraction in dyne cm-1
100	1	0	1	3,1	3,7	0	9,6	9,6	0,141	2400	0,134	4500
	2	0	1	3,2	3,8	0	9,6	9,6	0,145	1200	0,137	3500
120	3	1,1	1,7	2,8	3,7	8,8	18,0	26,8	0,137	3500	0,129	6300
	4	1,1	1,7	2,7	3,6	8,8	18,2	27,0	0,137	3500	0,129	6300
140	5	2,2	2,0	3,2	4,9	17,6	24,5	42	0,131	5500	0,121	9500
	6	2,2	2,0	3,2	4,9	17,6	24,5	42	0,132	5100	0,121	9500
160	7	3,5	2,5	3,6	4,9	28	27	55	0,127	7100	0,117	11500
	8	3,5	2,5	3,6	4,9	28	27	55	0,128	6400	0,120	10000
180	9	4,7	3,0	3,8	4,6	37,6	29	68	0,122	9100	0,112	14100
	10	4,7	3,0	3,8	4,6	37,6	29	68	0,122	9100	0,114	13000
200	11	6,1	3,0	3,3	3,8	48,8	27,6	76	0,119	10500	0,112	14100
	12	6,1	3,0	3,3	3,8	48,8	27,6	76	0,118	10900	0,112	14100
220	13	11	2,5	3,6	3,7	88	21	109	0,104	19000	0,102	20600
	14	11	2,5	3,6	3,7	88	21	109	0,105	18500	0,103	19900

Fibre length = 1,5 mm T_0 = 0,150 T_0' = 0,171 Temp: 6,5° C.
M_0' = 3 g M_0 = 10,1 S_0 = 17700 dyne cm^{-1}.

from the inverse proportional changes in length and cross-section, we have not considered it necessary to transform the stiffness measured to elasticity moduli. Thus the directly measured stiffness is more suitable as material constant.

When Gasser and Hill (1924) with similar technique found that the elasticity modulus of contracted muscle was 16 times that of resting muscle, they interpreted this increase as being due to the contraction process only, disregarding the possible influence of the increase in tension on stiffness. Buchthal (1942), however, showed that stiffness in resting muscle increases with tension and that the main part of stiffness due to contraction is caused by the increase in tension and only in a lesser degree by the contraction process proper.

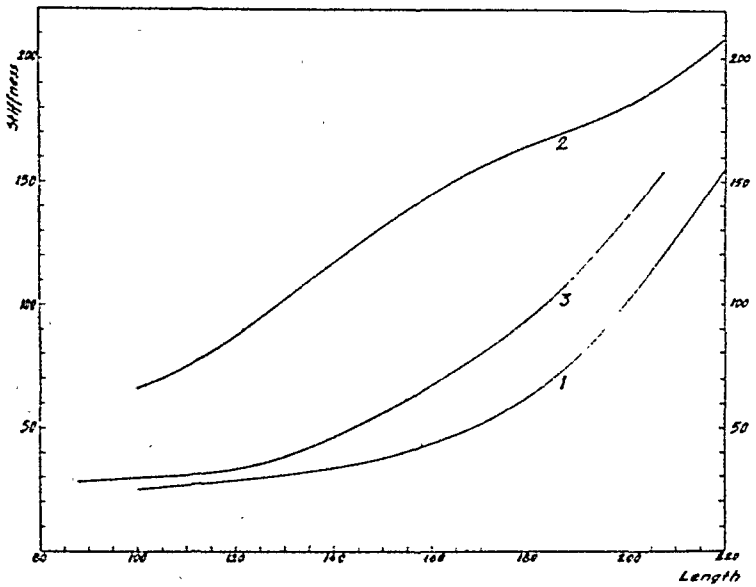

Fig. 22. Length-stiffness diagram of a cardiac muscle fibre at rest, during isometric and release contraction.
(1) length-stiffness diagram at rest.
(2) length-stiffness diagram during isometric contraction.
(3) length-stiffness diagram of release-contraction (fibre released to the same tension as at rest).
Abscissa: length of the fibre (equilibrium length = 100).
Ordinate: stiffness in arbitrary units.

In the following, stiffness at rest and during contraction are compared both as function of length and tension, the latter being a better expression of changes in stiffness due to the contraction process.

Dynamic Stiffness.

Vibration experiments.

Stiffness as function of length at rest. Already at length 100 a stiffness is present at rest which increases in a curve concave upwards. the course of which is similar to that of the length-tension diagram i. e. till length 180 the gradient increases gradually and then rises steeply (1. fig. 22). The stiffness present at length 100 where tension is zero is an expression of a certain structure present in the unstretched muscle.

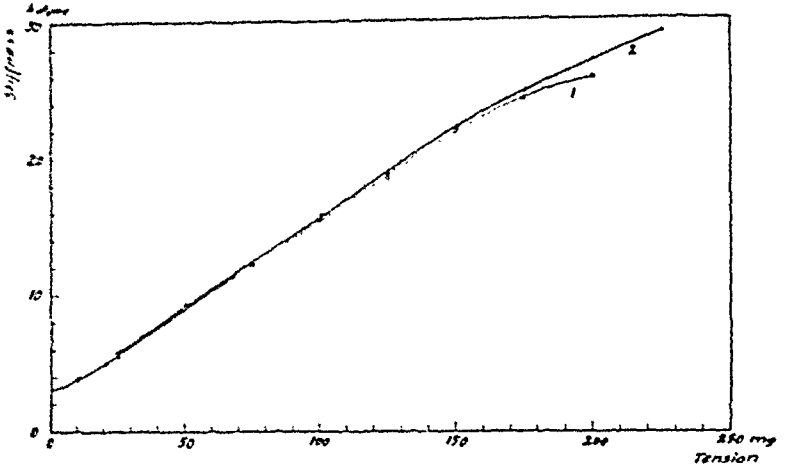

Fig. 23. Stiffness as function of load at rest and during isometric contraction, the curve for release-contraction coinciding with that at rest.
(1) stiffness-tension diagram at rest and during release-contraction.
(2) stiffness-tension diagram during isometric contraction.
Abscissa: tension in mg.
Ordinate: stiffness in dyne cm^{-1}.

Stiffness as function of tension at rest. The increase in stiffness is proportional to the increase in tension i. e. the course of the curve is linear although there is a deviation at the beginning and end of the stiffness-tension diagram towards a less steep gradient (1. fig. 23). The initial deviation of the gradient from linear course is due to a coarse adjustment of fibre elements, which occurs before stiffness is an expression of minute structure.

Stiffness in release during contraction to the same tension as at rest. Length-stiffness curves during rest and release contraction have a parallel course (3. fig. 22). Stiffness as function of tension is identical at rest and during release contraction and coincides with curve 1. fig. 23.

Stiffness in isometric contraction.

When comparing stiffness of the contracted fibre (st_c) with that of the resting fibre (st_r) at the same length the former is essentially higher (2. fig. 22). At length 100 the proportion $st_c : st_r = 3.5$ and remains constant till length 140 then slowly decreases, being 2.6 at length 170 and 1.85 at length 200. It falls further but in

no case does it reach 1, the lowest value being 1.3 at length 240. The shape of the length-stiffness diagram is similar to that of the corresponding length tension diagram. In the beginning the curve rises steeply and then converges towards the resting curve at the same length where the length-tension diagram of the contracted fibre approaches that of the resting fibre.

Stiffness as function of tension in isometric contraction has the same course as that at rest and release contraction, although the first tendency of the rest diagram towards a less steep course at low tensions is not marked in contraction (2. fig. 23). *Furthermore, the stiffness-tension diagrams at rest and release contraction coincide on the same straight line* within the range where tension can be compared under the different conditions. At high tensions the diagram has a less steep course i. e. it deviates from the straight line so that stiffness increases less steeply than tension.

If muscle lengths are compared at the same tension in length-stiffness and stiffness-tension diagrams the muscle is found to be longest at rest, shorter in release contraction and shortest in isometric contraction. The continuations of length-stiffness diagrams intersect the abscissa at the same point i. e. stiffness zero is obtained at about length 70.

Above length 70 (stiffness = zero) three different conditions are found in muscle where tension and stiffness are identical but length and cross-section vary considerably. In other words *stiffness in cardiac muscle is determined by the tension present* and not by length and cross-section as in isotropic elastic bodies.

A comparison of skeletal and cardiac muscle reveals essential differences. With the frequencies applied here, stiffness in the contracted skeletal muscle is always higher at the beginning of the length-tension diagram than at rest and attains the resting values only after a critical tension (yielding). Thus the stiffness-tension diagram during contraction deviates essentially from that at rest while those for cardiac muscle coincide. While in skeletal muscle stiffness can be used as an indicator of both presence and amount of contracting substance, this is not possible in cardiac muscle.

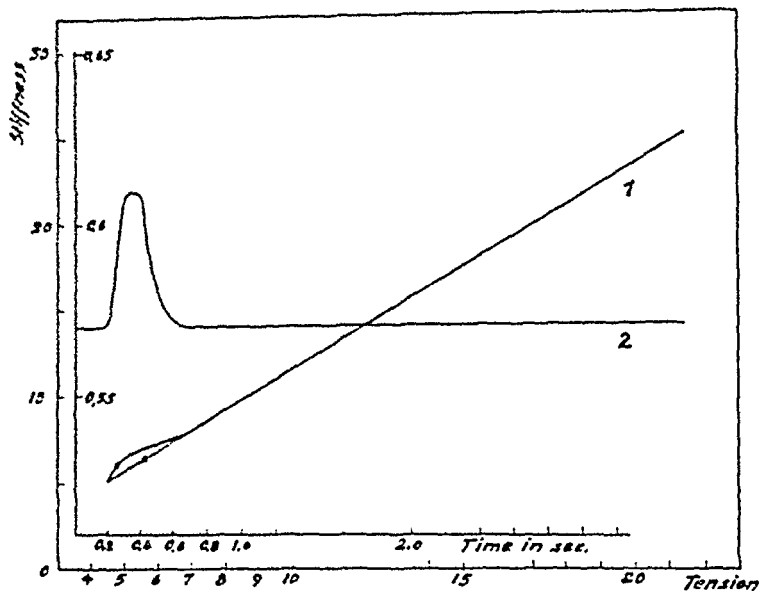

Fig. 24. Stiffness as function of tension and time during contraction.
(1) stiffness-tension diagram during contraction (the arrows indicate direction of stiffness-tension variations).
(2) stiffness in proportion to tension $\left(\frac{st}{y+b}\right)$ as function of time.
Abscissa (lower, belonging to curve 1) : tension in arbitrary units.
Abscissa (upper, belonging to curve 2) : time in sec.
Ordinate (left, belonging to 1) : stiffness in arbitrary units.
Ordinate (right, belonging to 2) : $\frac{st}{y+b}$

Semi-dynamic stiffness investigations.

When semi-dynamic stiffness is measured at the height of contraction the length-stiffness diagrams at rest and during contraction are identical with the corresponding curves from the vibration experiments. This applies also to stiffness-tension diagrams.

The stiffness-tension diagram in semi-dynamic contraction shows that at the beginning of contraction stiffness increases steeply, after which it has a linear course during both increase and decrease of tension (fig. 24). The gradient is the same as in the above mentioned stiffness-tension diagrams in vibration experiments. At the start of contraction there is an unproportionality between tension and stiffness. If these semi-dynamic diagrams are treated as dynamic stiffness deter-

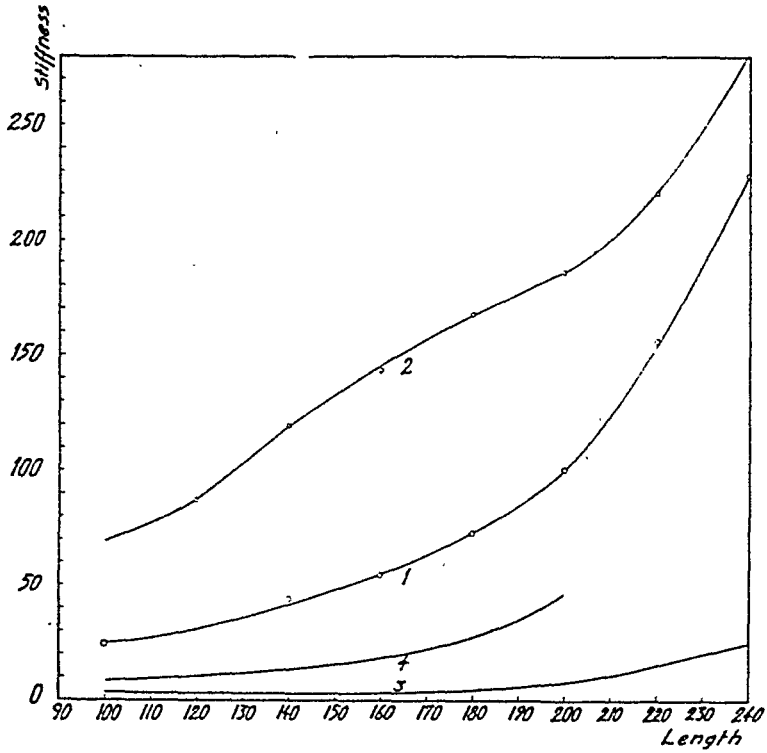

Fig. 25. Static and dynamic stiffness as function of length in cardiac muscle (mean curve).

(1) dynamic stiffness at rest.

(2) dynamic stiffness during isometric contraction.

(3) static stiffness at rest.

(4) static stiffness during isometric contraction (determined from release diagrams during contraction).

minations of single contractions in skeletal muscle have been treated by Buchthal and Kaiser (1944), we find that the proportion between stiffness and tension can be expressed by $\frac{dst}{dy} = F$, where F is a constant, dst the increase in stiffness and dy the increase in tension. As is seen from the stiffness-tension diagram (fig. 23), already at tension zero a certain stiffness is found in muscle. By extrapolation to stiffness zero we get a corresponding negative tension termed stiffness-tension (b). The stiffness-tension diagram shows that b is the same in rest and contraction. The linear stiffness-tension dia-

gram can thus be expressed by the equation $st = (y + b) F$, from which follows $F = \frac{st}{y + b}$.

Studying the coefficient F more closely during contraction, we find that at the start of contraction F rises steeply reaching a maximum after about 0.15—0.2 sec, returning to its original value after a further 0.3—0.4 sec. From this it is seen that stiffness at the start of contraction rises relatively more rapidly than tension, a fact also valid for skeletal muscle (Buchthal and Kaiser 1944). According to these authors this increase in stiffness is not due to extrinsic tension but to intrinsic tension caused by the interaction of contracting and non-contracting shunting substances.

Proportion between static and dynamic stiffness.

The static stiffness determined from the gradient of the length-tension diagram at rest is considerably less than the corresponding dynamic stiffness (fig. 25). At length 100 the dynamic stiffness is 10 times the size of the static, after which it increases so that at length 120 it is 13 times as high, and at lengths 160—200, 16 times as high. At higher elongations it decreases towards the value found at equilibrium length. This is in contrast to skeletal muscle where the proportion between dynamic and static stiffness at rest is approximately constant in the ratio 2:1.

The variations of $\frac{\text{static stiffness}}{\text{dynamic stiffness}}$ as function of length could be due to the fact that at equilibrium length part of the tension goes to mechanical straightening out of the muscle. At high degrees of stretch, static conditions are not achieved after the relatively short consolidation time of half-an-hour which generally elapses before stiffness is recorded, so that stiffness is not completely static.

Static and dynamic stiffness cannot be compared during contraction. In *skeletal muscle* the reversible release diagram is used as an expression of static stiffness. Due to the higher consolidation time, release diagrams cannot be regarded as static in *cardiac muscle* and therefore cannot be used as a basis for comparison. The curve of the isometric maxima could be used as measure of static stiffness.

were it not for the fact that high viscosity makes it impossible to decide whether elastic locking is present or not. In spite of the above reservations we have determined static stiffness partly from release diagrams, partly from curves for the isometric maxima. Dynamic stiffness during contraction in proportion to static stiffness from release diagrams varies in the same way as the corresponding stiffness in the muscle at rest. The proportion is, however, relatively less as, due to viscosity, static stiffness is too high. Dynamic stiffness in proportion to static stiffness (determined from the gradient of the isometric maxima) behaves similarly except that the values are somewhat higher than at rest.

While the proportion between static and dynamic stiffness at rest is 1 : 2 in skeletal muscle it can amount to 1 : 16 in cardiac muscle. Viscosity, therefore, is of far more importance in cardiac than in skeletal muscle. An investigation of cardiac muscle with a frequency of 5 cycles per sec corresponds to an investigation of skeletal muscle at a much higher frequency, i. e. frequencies applied here provide more information on the dynamic properties in cardiac muscle than in skeletal muscle. Consolidation time has still, however, considerable influence. If frequency is increased from 8 to 13 by decreasing the mass of the oscillating system, stiffness increases by 30 per cent. A further analysis of purely dynamic properties disregarding the influence of consolidation time would thus require still higher frequencies.

Elasticity moduli at rest and during contraction.

The elasticity moduli as function of cross-section loading have been calculated from stiffness and relative length (fig. 26). Cross-section × length is regarded as being constant at rest and during contraction at different degrees of stretch (constant volume). E-moduli at low cross-section loading coincide for muscle at rest, during isometric and release contraction. With increasing load the moduli for resting muscle are higher than those during release contraction which in turn exceed values for the isometrically contracted fibre. In the example given in fig. 26, at cross-section loading 60, the elasticity modulus for the resting muscle is 50 and for release contraction 20

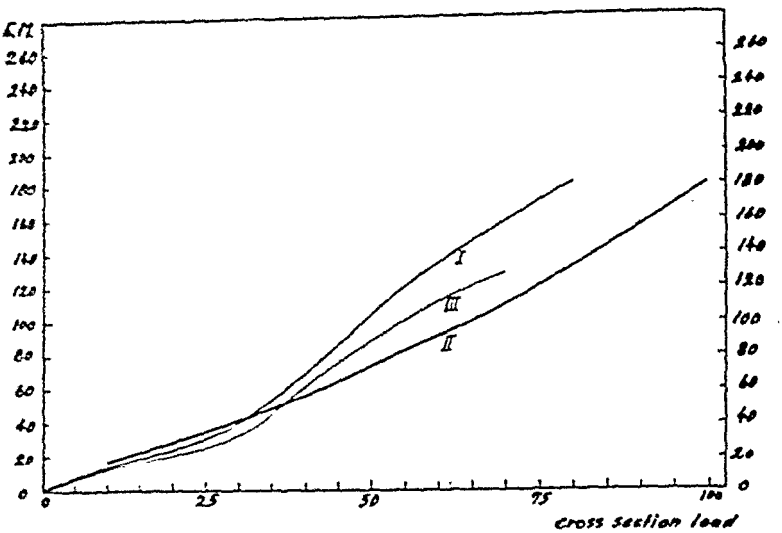

Fig. 26. Elasticity modulus of cardiac muscle as function of cross-section load.
(1) resting fibre.
(2) isometrically contracted fibre.
(3) release during contraction to the same tension as at rest.
Load and elasticity moduli in arbitrary units.

per cent higher than that for the isometrically contracted fibre. At still higher loading the differences decrease. As mentioned before the moduli are not the best means of characterizing structural properties of highly elastic bodies.

Absolute values of elasticity moduli in cardiac muscle.

In a number of experiments apart from tension and stiffness the cross-section of the fibre was determined by the procedure described on page 32. Elasticity moduli were computed according to formula 10 page 30. In the resting fibre at equilibrium length they amount to 0.001 kg per mm^2 and at maximal elongation to 0.01 kg per mm^2. These moduli are denoted in the following by E-min and E-max respectively. When transforming the values given in kg per mm^2 to the c. g. s. system by multiplying by $981 \cdot 10^5$, we get E-min $= 0.0981 \cdot 10^6$ and E-max $= 0.981 \cdot 10^6$, or in round figures $(0.1 \text{ and } 1) \cdot 10^6$ dyne cm^{-2} respectively. The corresponding dynamic elasticity moduli are $(0.8 \text{ and } 10) \cdot 10^6$ dyne cm^{-2}.

In the following table a comparison is performed of static elasticity moduli from skeletal muscle, cardiac muscle, myosin and caoutchouc.*)

Table 3.

Preparation	Longitudinal elasticity modulus in dynes × cm^{-2}	Author
strips from ventricle wall .	$(0{,}03—0{,}04).10^6$	Wöhlisch & Clamann 1936
homogenous ventricular muscle bundle	$0{,}1.10^6$	Lundin 1944
total skeletal muscle	6.10^6	E. Weber 1846
„ „ „	$9{,}4.10^6$	Wundt 1858
„ „ „	$(0{,}1—10).10^6$	Triepel 1902
„ „ „	$(2{,}7—3{,}9).10^6$	Bouckaert a. o. 1930
„ „ „	$(0{,}9—1{,}7).10^6$	Wöhlisch a. o. 1927
0,5 mm part of injured fibre	$(0{,}7—2{,}8).10^6$	Sichel 1934
Non-injured single fibre .	$0{,}5.10^6$	Buchthal 1942
Slightly vulcanized caoutchouc	10.10^6	K. H. Meyer 1940
Caoutchouc not specified .	5.10^6	Wöhlisch 1940
Myosin thread	20.10^6	H. H. Weber 1934

The majority of values give the E-moduli at equilibrium length. Elasticity moduli for cardiac muscle lie below those found for skeletal muscle. If the elasticity moduli for cardiac muscle are compared with those found by Buchthal (1942) for m. semitendinosus, the latter are 5 times as high as the former. With the same technique as used here, Buchthal found a dynamic stiffness in skeletal muscle of $(0.81 \pm 0.11) \cdot 10^6$ dyne cm^{-2} which coincides with our value of $0.8 : 10^6$ (stiffness at equilibrium length). Frequency, however, in Buchthal's experiment is 5 while in ours it is 8

*) In the table given by Buchthal (1942) the factor used for transformation of the elasticity moduli in kg per mm^2 to the c. g. s. system is incorrect. Therefore a new comparison is given here.

vibrations per sec. If we reduce stiffness in cardiac muscle to the lower frequency used in skeletal muscle, the dynamic stiffness is likewise lower than that in skeletal muscle.

Our experiments show the E-modulus to be 3 times higher than that found by Wöhlisch and Clamann (1936) for strips of cardiac muscle and for the whole ventricle. This is valid for both E-min and E-max values, the latter being 10 times higher than E-min values. The difference is too great to be due to errors in measuring methods. Wöhlisch and Clamann worked with strips of cardiac muscle several mm long and some mm^2 in cross-section cut from the wall of the ventricle. Because of the different course of the muscle fibres within the ventricle wall in such a preparation, only part of the fibres, less than 50 per cent, go from end to end, the remainder may be more or less transverse to the longitudinal axis of the preparation. It is mainly the end-to-end fibres which determine tension during extension of the fibre. Tension should thus be originated by a number of fibres with a cross-section less than half that of the preparation used. Wöhlisch and Clamann's E-moduli are at least 50 per cent too low. It is surprising that the elasticity moduli found by Wöhlisch for the total ventricle and strips are identical. There are, however, a number of interfering factors difficult to control when determinations are made on the ventricle as a whole.

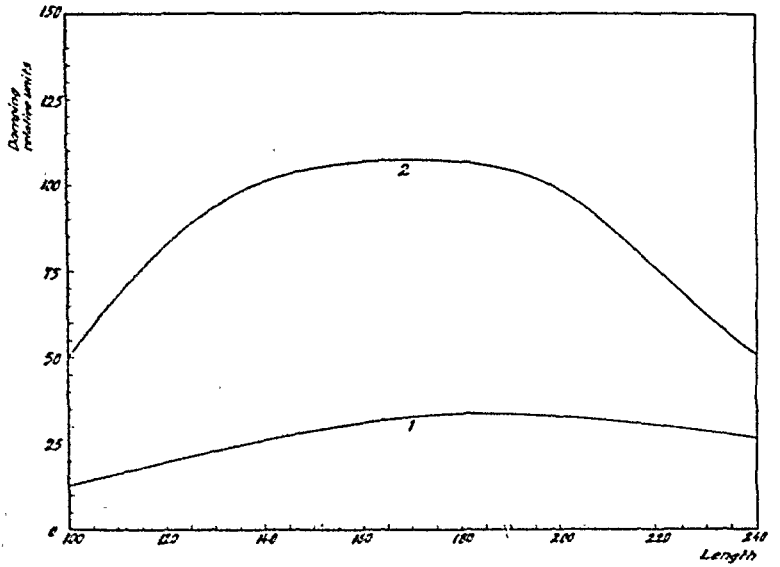

Fig. 27. Damping constant as a function of length (mean curve).
(1) damping constant at rest.
(2) damping constant during isometric contraction.
Abscissa: length of fibre (equilibrium length = 100).
Ordinate: damping constant in arbitrary units.

Experiments on viscosity

Damping constant.

The damping constant (P) was measured at a frequency of 8—12 vibrations per second (method c. f. page 34).

P as a function of length at rest: (1. fig. 27) At equilibrium length the damping constant is 12 dyne cm^{-1} sec and rises evenly with increasing length to reach about 30 at length 200. Stiffness in the experiments in question varies from 1000—10,000 dyne cm^{-1}.

The damping constant as function of length in isometric contraction (2. fig. 27) lies considerably above the curve of resting muscle. At length 100 the damping constant is 4 times as high as at rest increasing up to length 160—170 where it is 2.5 times higher than at length 100 and 3.5 times higher than P at rest. At still higher lengths P during contraction decreases to become, at the maximal length attained, about equal to that at length 100, and twice the size of P in resting muscle at the same lengths.

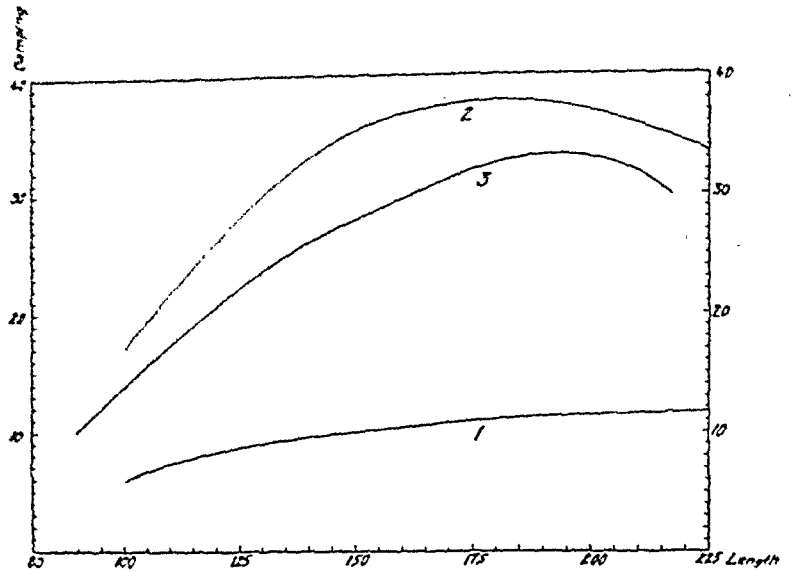

Fig. 28. Damping constant as function of length (mean curve).
(1) damping constant at rest.
(2) damping constant during isometric contraction.
(3) damping constant during release-contraction (release to the same tension as at rest).
Abscissa: length of the fibre (equilibrium length = 100).
Ordinate: damping constant in dyne cm^{-1} sec.

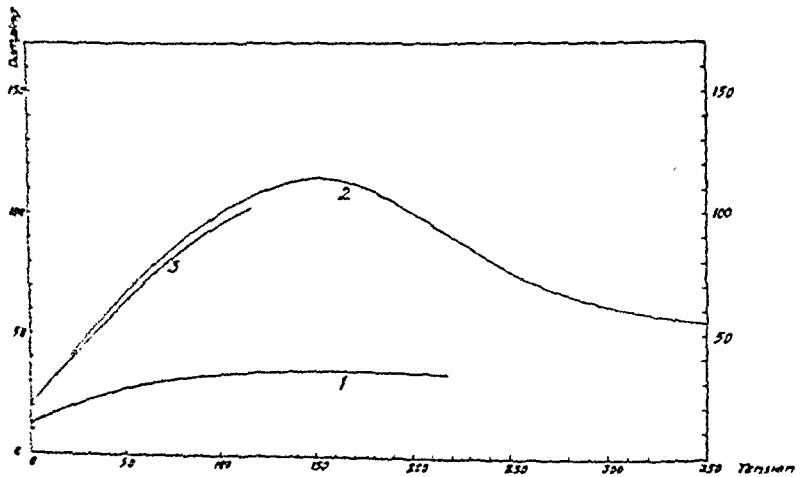

Fig. 29. Damping constant as function of tension (mean curve).
(1) damping constant at rest.
(2) damping constant during isometric contraction.
(3) damping constant during release-contraction (release to the same tension as at rest).
Abscissa: tension in arbitrary units.
Ordinate: damping constant in arbitrary units.

The damping constant as function of length in release contraction (3. fig. 28) is somewhat lower than in isometric contraction when comparing at the same lengths, but it lies appreciably above the curve for the constant at rest, at equilibrium length being about 1.5—2 times higher than that for the resting muscle.

The damping constant as function of tension. When comparing rest and contraction, stiffness values were referred to the same tension, as tension as such causes essential changes in stiffness. Similar considerations can be applied to determinations of damping constant. P is likewise influenced by tension and if we want to know *the influence of contraction proper, rest and contraction must be referred to the same tension.* As stiffness and tension are proportional the stiffness damping diagrams will also be proportional. At slight tensions the proportion between P in isometric contraction and P at rest is 2 : 1. With increasing tension it rises to 3 : 1. At still higher tension the difference between isometric contraction and rest becomes less and amounts to 2 : 1 at the maximum.

The damping constants of release contractions coincide with those for isometric contractions when compared at the same tension. When tension is zero P for release contraction is 1.5 times higher than that at rest.

Proportion between elastic and viscous stiffness.

Viscous stiffness is calculated from the product of the damping constant and angular velocity (formula 5 page 35). The elastic resistance is determined by pure elasticity (st_e) and by viscosity (st_v). The damping constant is only a relative measure of the viscous forces present in the system. In the following the viscous stiffness and its contribution to total stiffness is accounted for, giving us a gauge of the non-elastic resistance at the different frequencies examined. At rest and equilibrium length the proportion between st_e and st_v is 4. At maximal lengths it increases to 12—15. Elastic stiffness as function of length in contraction corresponds to the curve of viscous stiffness up to length 170—180 i. e. $st_e : st_v = 4 : 1$. Above this length the proportion increases reaching 10 at maximal

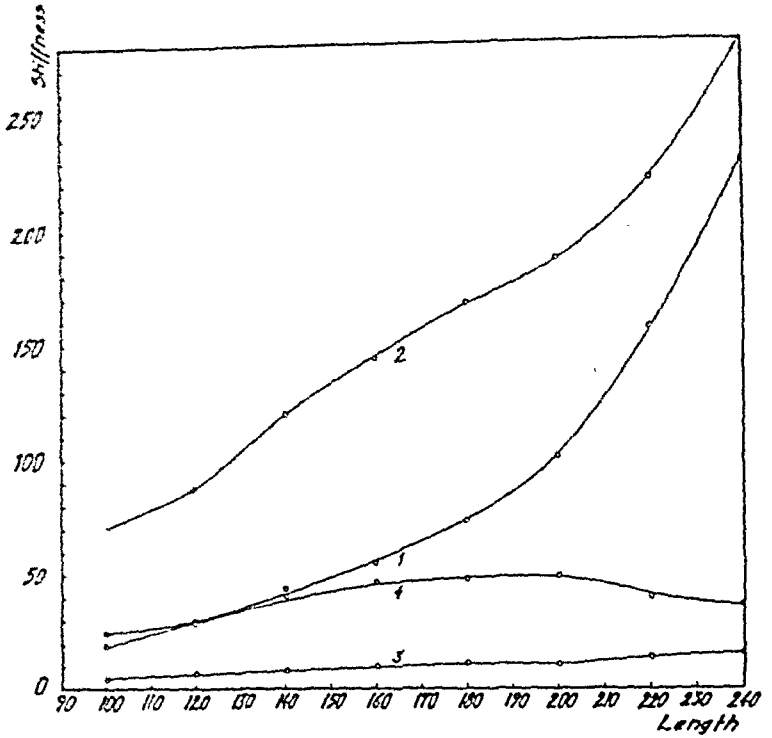

Fig. 30. Elastic (st_e) and viscous (st_v) stiffness in cardiac muscle as function of length.
(1) length - st_e diagram at rest.
(2) length - st_e diagram during isometric contraction.
(3) length - st_v diagram at rest.
(4) length - st_v diagram during isometric contraction.

lengths and the curves for st_v in contraction and rest approach each other.

The effect of frequency and tension on damping at the same length is determined by varying the mass of the oscillating system and with it frequency (formula 1 page 29). In one series frequency is 7—8 cycles per sec in the other 12—13 cycles per sec, being thus 60 per cent higher in the latter case. Damping decreases on the average about 25 per cent with this increase in frequency, while stiffness increases correspondingly.

The above facts show that there is a certain relation between tension and damping; the same damping constant is found at the same tension irrespective of whether the latter is originated by isometric

or release contraction. On the other hand, *both during isometric and release contraction, the damping constant lies considerably above values found in the resting fibre when referred to the same tension.* This difference indicates that the contribution of the contractile processes to damping is much larger than that of the increase in tension.

As previously mentioned a considerable time has to elapse after stretch of cardiac muscle before it is consolidated and suitable for further experiments. The long consolidation period indicates that viscosity is not evenly distributed over the whole muscle e. g. shunted to elasticity, as then only an instantaneous increase in extra-tension would be found during extension proper, which would disappear immediately after extension, and tension would establish itself on a new static level. Experiments with different vibration frequencies resulting in falling damping with rising frequency indicate that a great part of the viscosity lies in series with the elastic elements of muscle. Viscosities lying in parallel with the elastic elements should give equal damping constants when different frequencies are applied.

When damping constant in cardiac muscle is compared with that of skeletal muscle no essential differences are found for the resting fibre, while during isometric contraction and release contraction the increase of damping constant with increasing length reaches a maximal value much earlier in skeletal than in cardiac muscle. Already at length 100 maximal values for P are found in skeletal muscle. It is noticeable that at lengths where damping constants have their maximum, extra-tension both for skeletal and cardiac muscle attains its highest value.

Elastic after-effect.

Like damping, *elastic after-effect* is an expression of viscosity. It has therefore been studied partly in experiments with rapid elongations, partly in stretch experiments of long duration.

Experiments with rapid elongations at rest show that immediately after stretch tension falls rapidly in less than 0.05 seconds to 30—40 per cent of the initial tension at length 100 (I, fig. 31). At higher

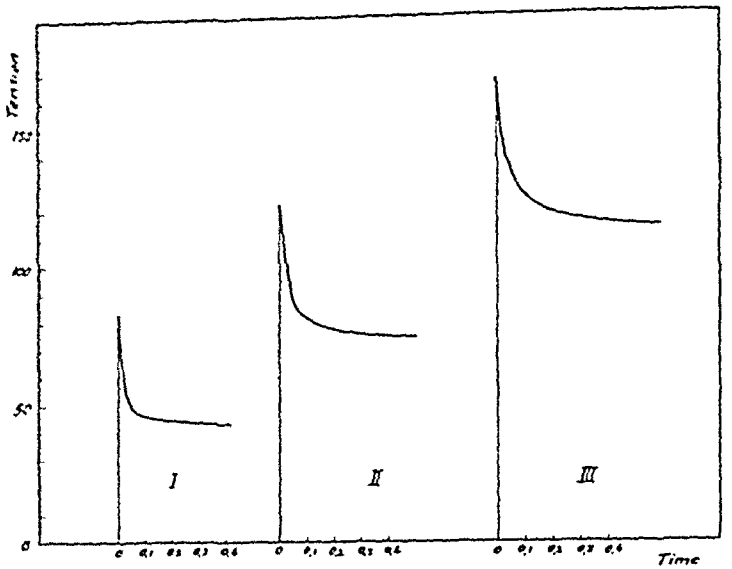

Fig. 31. Course of consolidation after rapid stretches of cardiac muscle at different lengths.
I. stretch from length 100 to 120.
II. „ „ „ 140 to 160.
III. „ „ „ 180 to 200.
Abscissa: time in sec.
Ordinate: tension in arbitrary units.

elongations (length 180) (III, fig. 31) the fall in tension amounts to only 20—30 per cent of the initial tension. For the following 0.1—0.2 seconds the gradient of tension decrease is less steep and becomes still less so in the following time period. The fall in tension is not consolidated in the minute the experiment lasts. When compared with initial tension the gradient of tension decrease is less, the higher the elongation.

During contraction just as at rest *sudden elongations* after a tension increase show a rapidly falling tension. The consolidation course can here be followed only with difficulty as the contraction tension of the muscle lasts only a short time, and changes in contraction tension interfere with the fall in tension due to consolidation. One second after elongation, however, tension reaches a value considerably above that for the isometric maxima at the lengths in question, thus indicating that consolidation is far from finished. Just as in

elongation of the resting fibre increase in tension is steeper, the higher the initial lengths.

Consolidation course after elongation observed during a longer period.

Experiments with rapid stretch at rest and during contraction show that consolidation is not complete after an observation period of 1 min. When observations are continued over a longer period consolidation is still incomplete after several hours. This consolidation course, like the initial course, can be divided into several exponential curves. Measurement of consolidation begins about 20 seconds after elongation so that here observations start at the third phase of consolidation registered at rapid elongations. This first relatively rapid relaxation lasts up to 10 minutes, after which comes the next phase lasting about half-an-hour and then the last unfinished phase which still goes on for 2—4 hours when the experiment cannot be continued for other reasons.

The fall in tension in the different experiments can amount to more than 80 per cent of initial tension up to length 150. At higher elongations the decrease in tension is relatively higher, being more than 90 per cent of initial tension. In the last case the slow consolidation process contributes relatively more to relaxation.

Relaxation of the resting and contracting muscle also gives information on viscous conditions. It shows that, as already mentioned in length-tension experiments, the fall in tension is similar during rest and contraction and, as in stretch experiments, changes in tension are steeper at greater initial lengths. It also shows that there is no elastic locking in cardiac muscle, the curves in question being caused by consolidation.

Comparison between viscous stiffness in skeletal and cardiac muscle.

A comparison of skeletal and cardiac muscle is seen in Table 4.

For skeletal muscle in a range of stretch 100—200 at rest, $st_e : st_v$ varies between 2 and 8. For cardiac muscle the variation lies between 4 and 15. Apart from the fact that frequency enters the calculation

Table 4.

	Rest $st_e : st_v$		Contraction $st_e : st_v$	
	Equilibrium length	High elongation	Equilibrium length	High elongation
Skeletal muscle, frequency 5 cycles per sec. (Buchthal 1942)	2.2	8	6	8
Cardiac muscle, uncorrected, frequency 10 cycles per sec.	4	15	4	10
Cardiac muscle, corrected to frequency 5 cycles per sec.	2	8	2	5

of viscous stiffness, it must be remembered that stiffness as well as damping constant is dependent on frequency, and viscous damping must be regarded as placed in series with elasticity. The value for skeletal muscle is derived from experiments with a frequency of 5 cycles per sec while that for cardiac muscle from experiments with a frequency of 10 cycles per sec. A direct comparison is thus impossible without knowing the dependence of stiffness on frequency. The relatively high viscous stiffness in skeletal muscle can however be explained as being due to the *different* frequency applied. As regards the consolidation experiment it is surprising that no essentially greater damping constant is found for cardiac than for skeletal muscle.

From the above we can conclude that frequency dependence of stiffness is more pronounced in cardiac than in skeletal muscle. The difference between static and dynamic stiffness is much higher in cardiac than in skeletal muscle. Both react similarly as regards the rapid consolidation course *while over the longer consolidation period the viscosity of the heart is considerably greater*. While there is conformity between skeletal and cardiac muscle at 5—10 cycles per sec, semi-dynamic frequencies show essential deviations which may be of importance for the function of the heart.

If viscosity in cardiac and skeletal muscle is compared during contraction it is seen to be higher in both cases than at rest. Viscosity in release contraction in cardiac muscle coincides with viscosity for isometric contractions when compared at the same tension. In contrast to this, damping in release contraction in skeletal muscle is higher than that in isometric contraction. This result emphasizes that there is no principle difference between isometric and release contraction in cardiac muscle, a difference which is pronounced in skeletal muscle and which is due to the elastic locking mentioned above.

A comparison between st_e and st_v for skeletal and cardiac muscle corrected to the same frequency shows that viscous resistance affects cardiac muscle more than skeletal muscle during contraction, while there is no essential difference at rest.

Excitability during consolidation of cardiac muscle.

Following rapid stretch it is often found that, after the initial increase and following rapid fall in tension, the muscle goes into strong contraction which persists and corresponds to a contracture-like condition (fig. 32). After some time weak spontaneous contractions of short duration can be superposed on this "contracture" resembling the flat peaks of normal contractions (IV. fig. 32). The curves remind us of Otto Frank's "Gruppenbildungen und tonusartigen Contraktionen" (1898) which appeared after sudden increase in pressure in the ventricle.

After extension of the muscle to high length (180—200) a fall in tension occurs which is relatively slow for several minutes. In this period the muscle is refractory even for strong stimuli. During consolidation the muscle regains its excitability when it reaches a certain tension level which varies with initial stretch. *The return of excitability is accompanied by a rather striking change in gradient of consolidation course.* Both return of excitability and the rapid tension fall indicate that a characteristic change in the fibre occurs at this moment. This course of consolidation has been observed at sudden stretches as well as at elongations of longer duration. In terms of Buchthal's molecular interpretation the disappearance

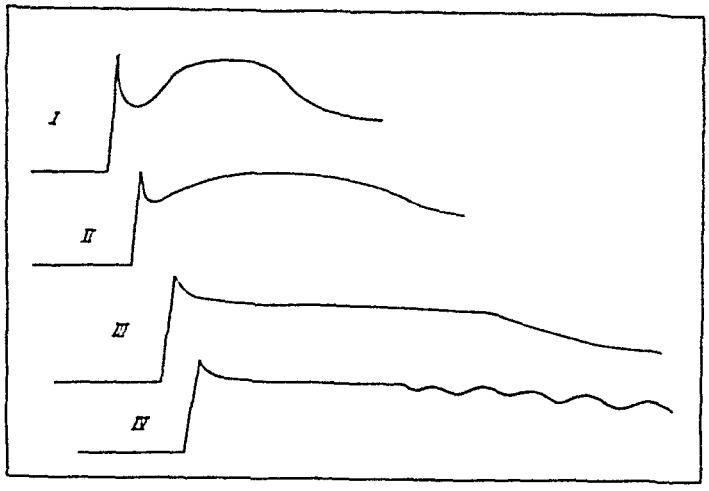

Fig. 32. Elongation of fibre followed by a contracture-like condition.
I. stretch from short length.
II. „ „ medium „
III and IV stretch from high length (IV shows superposed small spontaneous contractions.).

of excitability can be explained by the extreme straightening out of the contractile substance which prevents initiation and propagation of contraction over the minute structure element. When the contractile elements have attained a lower tension during consolidation, the result is a smaller mutual distance of the molecule links and contraction can be brought about. It may be mentioned that contraction tension cannot exceed the critical tension at which excitability returns (see IV fig. 32). Thus the strength of contraction extra-tension increases as the resting tension falls during consolidation, so that contraction extra-tension and tension at rest have the same top limit throughout. Consolidation can be accelerated if the muscle is allowed to contract.

The physiological importance of viscosity.

The high viscosity of cardiac muscle causes an abrupt fall in tension with release and a strong increase in tension with rapid stretches. The latter implies that the resistance of the muscle to rapid changes in length is considerable and, from many points of view, is an im-

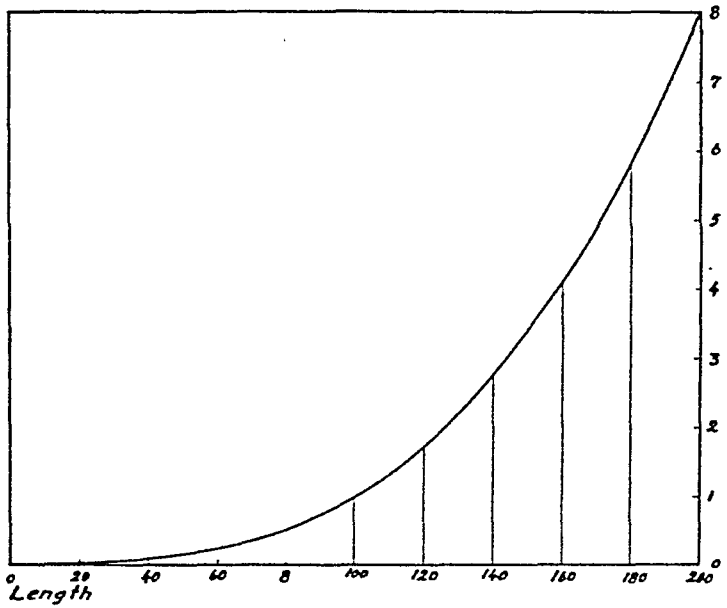

Fig. 33. Changes in heart volume due to shortening of the muscle fibre.
Abscissa: length of fibre in arbitrary units.
Ordinate: volumes at different lengths, arbitrary units.
Areas between vertical lines represent volumes ejected by respective shortening of the fibre.

portant property. *On the other hand can the fall in tension during release contraction be regarded as physiologically important?*

During systole the heart contracts isometrically in the beginning and the pressure curve rises steeply. When the valves open the rise in pressure continues for a short period. The muscle shortens and tension falls, following a curve with a gradient which lies near the length-tension curve for release contraction. This implies a greater decrease in duration of the systole than would occur if the fall in tension were to follow the curve of isometric maxima. The shortening of the systole may mean an improvement in the efficiency of the heart. If the rate of shortening in muscle is considered constant it follows that, in the initial phase of ejection, more blood per time unit is driven out than in the later phase as the heart volume decreases in inverse proportion to the third power of the degree of stretch. Thus the stroke volume increases less than proportionally with the degree of shortening. When pulse frequency

falls proportionally with shortening length, minute volume, which is a product of pulse frequency and stroke volume, decreases with increasing stroke volume. The diagram in fig. 33 shows the changes in volume during contraction in cardiac muscle, the shape of the heart being simplified to a sphere. It can be seen from the diagram that, with a shortening from length 200—180, 7 units of blood are driven out. With shortening from length 180—160 a further 5 units are driven out i. e. 12 units in all. 20 per cent further shortening adds only 3.5 units. Table 5 shows shortening of muscle, pulse frequency, stroke volume and minute volume.

Table 5.

Shortening	frequency	stroke vol.	min. vol.
20	6	7	42
40	3	7 +5 =12	36
60	2	12 +3.5=15.5	31
80	1.5	15.5+2.5=18	27
100	1.2	18 +1.3=19.3	24

When supposing the proportional decrease of pulse frequency with increasing stroke volume it follows that the minute volume decreases with larger stroke volume. A relatively high pulse frequency caused by decreased shortening of cardiac muscle would thus be of advantage. The relation between pulse frequency and stroke volume is in reality more complicated than supposed here, where the effect of extra-cardial innervation is not taken into consideration.

Theoretically high viscosity could also be of value in facilitating the diastolic blood flow from auricle and veins. Relaxation of the resting muscle follows a length-tension curve which in the main lies parallel with that of release contraction. This means that at the end of systole intraventricular pressure can be zero also at high degrees of stretch (dilated heart). Thus in-flowing blood in the beginning meets less resistance and consequently runs in more quickly, i. e. total work during diastole becomes less.

These properties become especially important in cardiac muscle with defects in aortic and pulmonary valves. Here an increased frequency is of value and steep fall of pressure facilitates a rapid filling from the auricle.

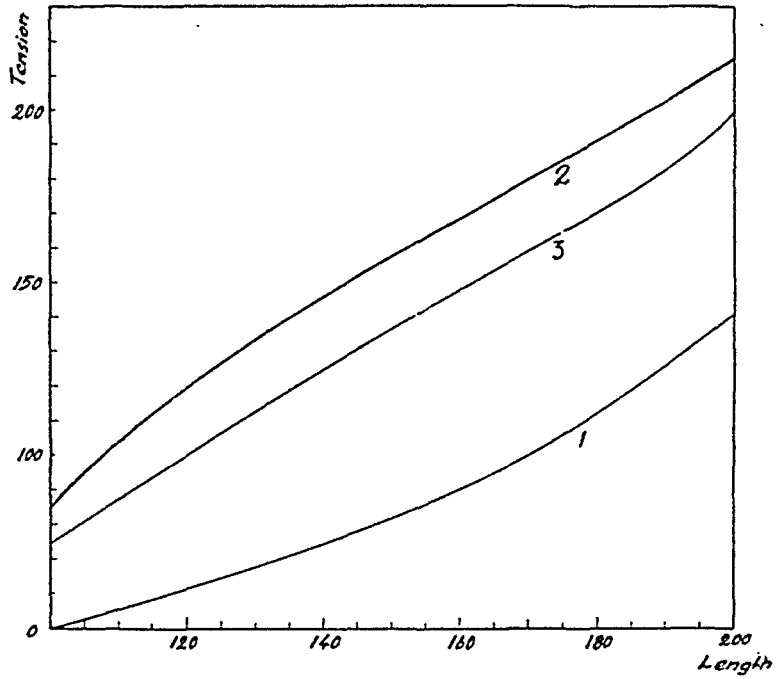

Fig. 34. Tension as function of length in cardiac muscle with and without acetylcholine (10^{-7} g/ml).
(1) length-tension diagram of the resting fibre with and without acetylcholine.
(2) length-tension diagram of fibre during isometric contraction without acetylcholine.
(3) length-tension diagram of fibre during isometric contraction after addition of acetylcholine.
Abscissa: length of fibre (equilibrium length = 100).
Ordinate: tension in mg.

Effect of acetylcholine and adrenalin on the mechanical properties of cardiac muscle.

The effect of acetylcholine and adrenalin on cardiac muscle has been the object of many investigations which have established that adrenalin increases and acetylcholine decreases the strength of contraction. With the method applied here to an analysis of mechanical properties, we have attempted to obtain information on the changes within the contractile substance causing the variations in tension originated by acetylcholine and adrenalin.

Length-tension diagrams (fig. 34 and 37) at rest show no difference whether adrenalin and acetylcholine are applied or not. Du-

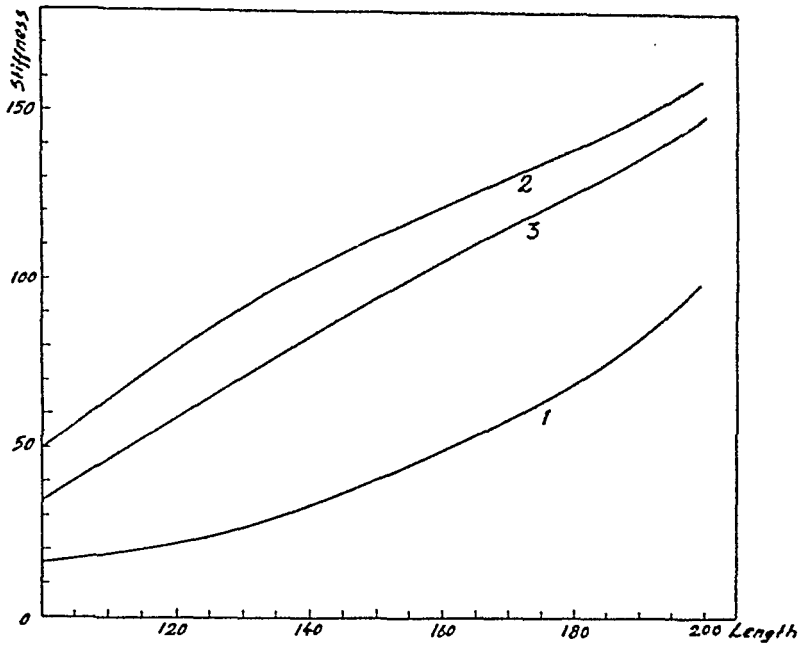

Fig. 35. Stiffness as function of length with and without acetylcholine.
(1) length-stiffness diagram of resting fibre with and without acetylcholine.
(2) length-stiffness diagram of fibre during isometric contraction without acetylcholine.
(3) length-stiffness diagram of fibre during isometric contraction after addition of acetylcholine.

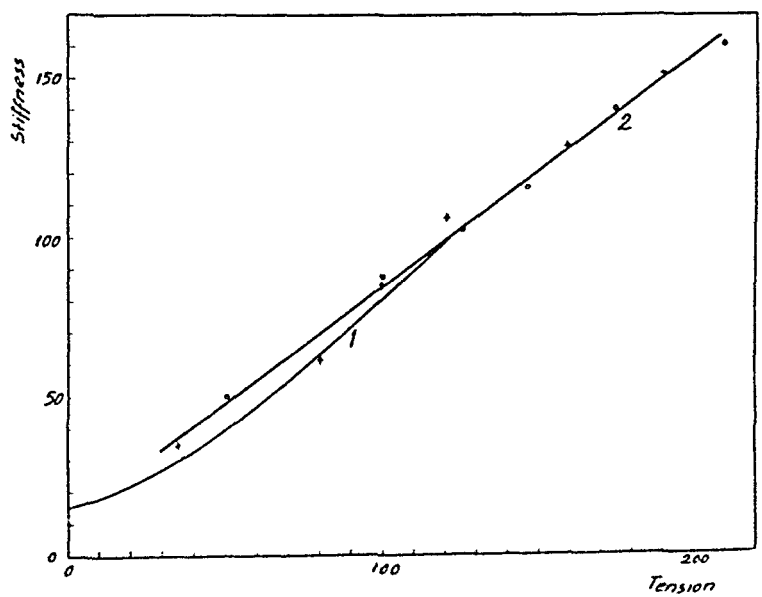

Fig. 36. Stiffness as function of tension in cardiac muscle with +—+—+— and without o—o—o acetylcholine.
(1) stiffness-tension diagram at rest with and without acetylcholine.
(2) stiffness-tension during isometric contraction with and without acetylcholine.

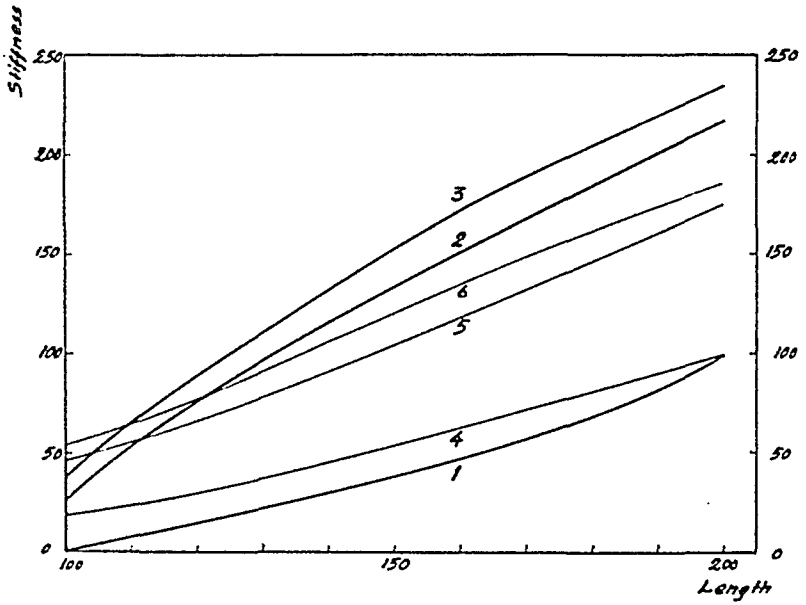

Fig. 37. Tension and stiffness as function of length in cardiac muscle with and without adrenalin (10^{-7} g/ml).

(1) length-tension diagram at rest with and without adrenalin.

(2) length-tension diagram during isometric contraction without adrenalin.

(3) length-tension diagram during isometric contraction after addition of adrenalin.

(4) length-stiffness diagram at rest with and without adrenalin.

(5) length-stiffness diagram during isometric contraction without adrenalin.

(6) length-stiffness diagram during isometric contraction after addition of adrenalin.

Abscissa: length of the fibre (equilibrium length = 100).

Ordinate: (to the left) stiffness in arbitrary units.

Ordinate: (to the right) tension in arbitrary units.

ring contraction, tension increases considerably with the addition of adrenalin ($1 : 10^7$ g/ml) in proportion to that in the normal muscle. The increase in extra-tension amounts to 30—40 per cent. After the addition of acetylcholine (1—5 : 10^7 g/ml) a corresponding decrease in tension is found during contraction which amounts to about 50 per cent of the contraction extra-tension of normal muscle.

Length-stiffness diagrams at rest after addition of adrenalin and acetylcholine are identical with those of normal muscle (fig. 35 and 37). During *contraction* however, stiffness is much higher after the

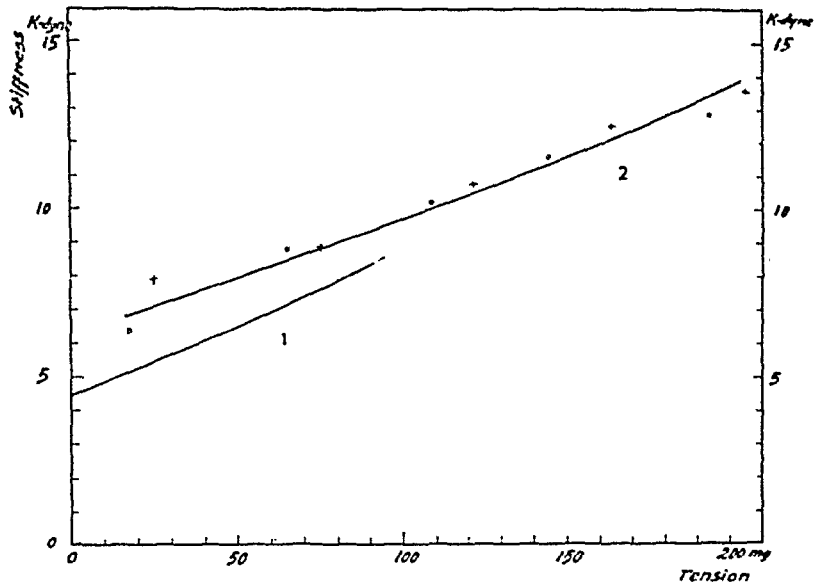

Fig. 38. Stiffness as function of tension in cardiac muscle with +—+—+— and without o—o—o adrenalin.
(1) stiffness-tension diagram at rest with and without adrenalin.
(2) stiffness-tension diagram during isometric contraction with and without adrenalin.

addition of adrenalin and much lower after the addition of acetylcholine than the corresponding stiffness in normal muscle. Here again the question arises as to whether this change in stiffness, originated by adrenalin and acetylcholine respectively, is due to tension or to other changes induced in the contractile material. The answer is found by comparing contraction tension with the same tension in the resting fibre i. e. in stiffness-tension diagrams. *Stiffness-tension diagrams at rest and during contraction coincide for muscles with and without drugs (fig. 36 and 38).*

Adrenalin and acetylcholine have thus no effect on the tension and stiffness in resting muscle. The so-called diastolic tension effect which among others was claimed to be caused by adrenalin (Eismayer and Quincke 1929, Ducret 1931) could not be observed in the present experiments.

Just as in normal muscle, stiffness investigations after application of adrenalin and acetylcholine have provided no evidence of structural changes in muscle during contraction. In normal muscle damp-

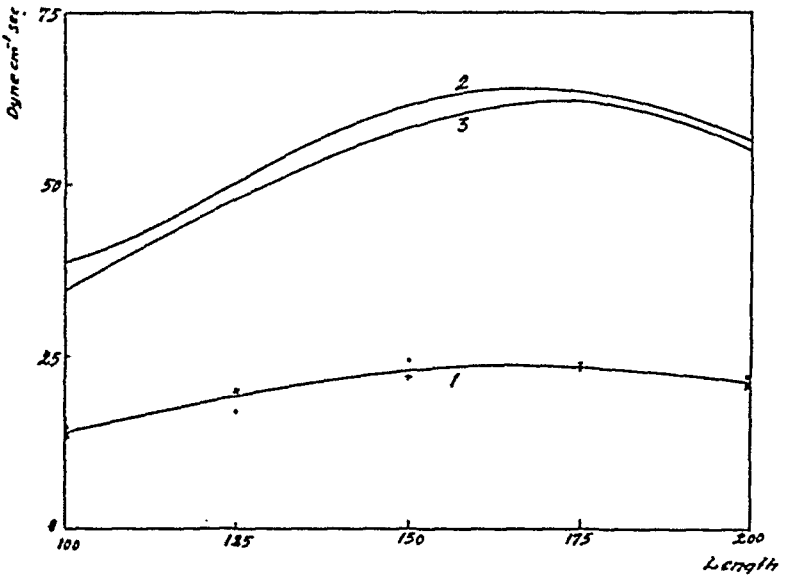

Fig. 39. Damping constant as function of length in cardiac muscle with and without acetylcholine.
(1) damping constant at rest with +—+—+ and without o—o—o acetylcholine.
(2) damping constant during isometric contraction without acetylcholine.
(3) damping constant during isometric contraction after addition of acetylcholine.
Abscissa: length of fibre (equilibrium length = 100).
Ordinate: damping constant in dyne cm^{-1} sec.

ing undergoes characteristic changes which are due to the contraction process itself. Therefore the *damping constant* was determined after the application of adrenalin and acetylcholine respectively. Damping constant at rest is identical for both normal and drugged muscles. As function of length during contraction it increases after application of adrenalin (fig. 41) and decreases after application of acetylcholine (fig. 39) compared with normal muscle. *On the other hand damping as function of tension as well as of stiffness coincides for drugged and normal muscles* (fig. 40 and 42).

From the above experiments it is seen that adrenalin and acetylcholine affect neither stiffness in proportion to tension nor the proportion $st_e : st_v$. On the other hand, as is well known, acetylcholine decreases and adrenalin increases extra-tension and duration of contractions.

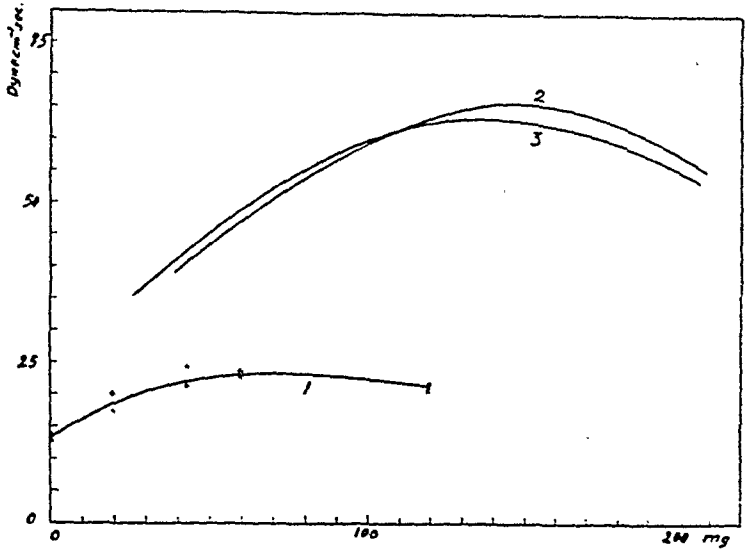

Fig. 40. Damping constant as function of tension in cardiac muscle with +—+—+ and without o—o—o acetylcholine.
(1) damping constant at rest with and without acetylcholine.
(2) damping constant during isometric contraction without acetylcholine.
(3) damping constant during isometric contraction after addition of acetylcholine.
Abscissa: length of fibre (equilibrium length = 100).
Ordinate: damping constant in dyne cm^{-1} sec.

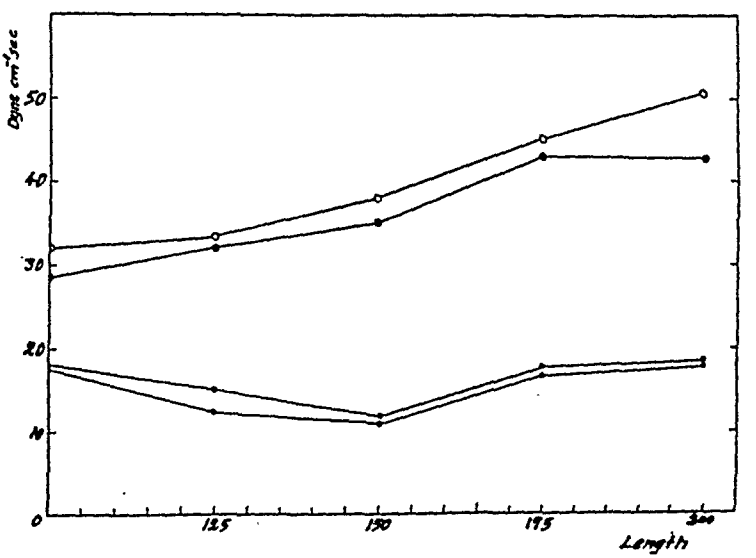

Fig. 41. Damping constant as function of length in cardiac muscle with o—o—o and without •—•—• adrenalin.
(1) damping constant at rest without adrenalin.
(2) damping constant at rest after addition of adrenalin.
(3) damping constant during isometric contraction without adrenalin.
(4) damping constant during isometric contraction after addition of adrenalin.

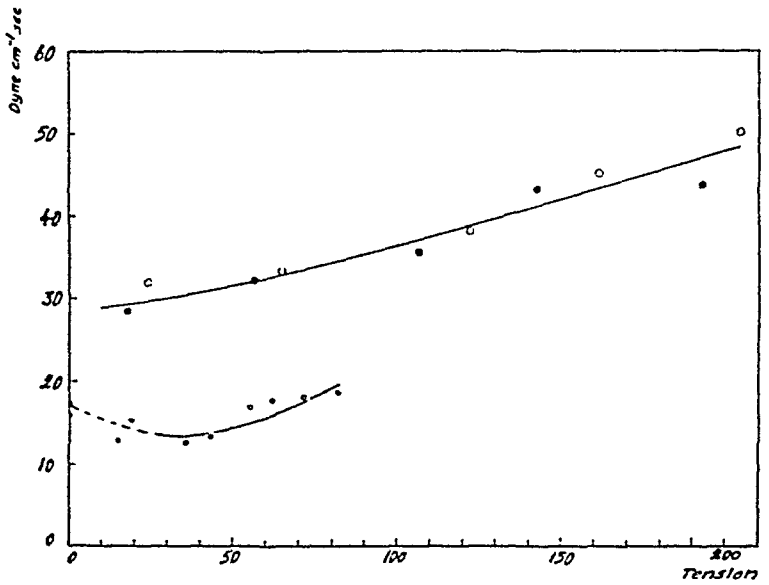

Fig. 42. Damping constant as function of tension in cardiac muscle with o—o—o and •—•—• without adrenalin.
(1) damping constant at rest with and without adrenalin.
(2) damping constant during isometric contraction with and without adrenalin.

With regard to stiffness and viscosity this negative result indicates that mechanical properties of the contractile substance are not affected by the drugs mentioned.

When a molecular interpretation of these results is attempted the negative findings indicate that the drugs do not affect the single links of the molecular chains (angular forces) which would induce a change in stiffness besides that in tension. It is more probable that the substance in question affects only the terminal links of the contractile chains and thereby alters the number of active molecules. The very small amount of these drugs able to affect tension likewise makes it more probable that not all links of molecule chains are evenly affected.

Summary

A modification of Buchthal's condensor myograph is used for registration of the changes in tension in a small bundle of cardiac muscle. A movable condensor plate alters its distance from a fixed condensor plate with increase of tension during contraction or with extension of the fibre. Changes in capacity are recorded by means of a high frequency arrangement with amplifier and electro-static oscillograph. The apparatus is also used to determine dynamic elasticity in vibration experiments.

The dependence of tension on length is investigated on muscle bundles containing few fibres only from the cardiac ventricle of a frog both in static and semi-dynamic experiments.

The length-tension diagram of the resting fibre up to length 120 rises somewhat more steeply than at further elongations. The tension then increases gradually with length up to length 180 where there is a steeper increase.

Total tension during isometric contraction (isometric maxima) shows a much steeper rise than tension at rest with increasing length up to length 170—200, after which tension at rest and during contraction approach each other. The curves never intersect and no "indifference point" is attained as in skeletal muscle. Irregularities in the curve of isometric maxima at higher elongations which could indicate processes comparable with yielding in skeletal muscle are not observed.

Extra-tension during contraction increases with increasing length and attains a maximum at length 175—200 where it is 6—8 times higher than at equilibrium length. In skeletal muscle this maximum is often reached already at equilibrium length.

Elongation and release during isometric contraction at a frequency of 0.5 cycles per sec give an increase in tension considerably above

that developed in the muscle stretched at rest and consolidated to the same length.

Release of the isometrically contracted muscle to the same tension as at rest (frequency 0.5 cycles per sec) shows a fall in tension considerably steeper than the curve of the isometric maxima. If the resting muscle is relaxed from the same initial length and stretched or relaxed to the same degree as during contraction a fall in tension is found which in the main is parallel with that of release of contraction. The similarity between the decrease in tension at rest and during contraction indicates that it is caused by incomplete consolidation and not by elastic locking, which explains the irreversibility of the curve of isometric maxima in skeletal muscle.

Length-tension diagrams of anisotropic (A) and isotropic (I) substance are constructed from the length-tension diagram of the total fibre (A+I) and the proportion between the lengths of A and I. The length of A and I is determined by micro-photography at rest and during contraction. The length-tension diagram of I is much steeper than that of A. If the relative increase of A and I are compared with reference to their respective equilibrium lengths, I is seen to extend relatively more than A. Thus A is 30 per cent less extensible than I. In skeletal muscle I is 20 per cent less extensible than A. During contraction at about equilibrium length the A substance shortens 10 per cent while the I substance extends 15 per cent. In all probability the I substance participates actively in the development of tension during contraction. For skeletal muscle the activity of I has been demonstrated by Buchthal in experiments which cannot be performed on cardiac muscle.

Static and dynamic elasticity are investigated, the static from the gradient of the length-tension diagram, the dynamic in vibration experiments with a vibration frequency of 8—12 per sec. In highly elastic bodies besides elastic elements, viscosity is found shunting the elasticity. Static elasticity is a measure of pure elasticity plus elasticity shunted by viscosity. While elasticity moduli are used as an expression of elastic properties in isotropic bodies they are less suitable in highly elastic anisotropic bodies. Stiffness $\left(\frac{\Delta \text{ tension}}{\Delta \text{ length}}\right)$ gives more correct information on the structural properties of the bodies

concerned. Stiffness expressed in dyne cm^{-1} is determined at rest, isometric contraction and release contraction.

Stiffness in proportion to tension at rest shows a more gradual course at the start, caused by a mechanical straightening out of the fibre substance, after which it increases linearly in proportion to loading.

During isometric contraction we have a similar course of stiffness with tension, and stiffness coincides with stiffness at rest when referred to the same tension.

Also in release contraction to the same tension as at rest, we find a stiffness coinciding with that in the isometrically contracting and resting muscle when referred to identical tension.

In contracted cardiac muscle in contrast to skeletal muscle we find no degree of stretch where stiffness decreases suddenly, a phenomonen caused by a sudden plastic increase of the equilibrium length, a so-called yielding. When fibre length is periodically altered with a frequency of 3—5 per sec, stiffness can be determined from the resulting changes in tension. At the start of contraction stiffness rises more rapidly than tension; otherwise stiffness and tension are proportional and coincide with resting values.

With a vibration frequency of about 10 oscillations per sec the mean proportion between dynamic and static stiffness at rest is 12 : 1. As the curve of the isometric maxima cannot be regarded with certainty as reversible, static stiffness cannot be definitely determined. If we suppose reversibility present, the proportion between static and dynamic stiffness is similar to that at rest.

Absolute static elasticity moduli at rest are minimally $0.1 \cdot 10^6$ (equilibrium length) and maximally $1.0 \cdot 10^6$ dyne cm^{-2}, the values in each case being 3 times more than those previously found. The dynamic moduli are at minimum $0.8 \cdot 10^6$ and at maximum $10 \cdot 10^6$ dyne cm^{-2}. Thus dynamic elasticity at the vibration frequency in question is represented by only 10 per cent elastic elements in the fibre.

Experiments with higher frequencies show that this is not an entirely dynamic value. To obtain this, vibration frequency must be considerably increased.

Viscosity in cardiac muscle expressed by the damping constant in the *resting muscle* increases evenly with increasing length and tension, and at high stretch it is twice as high as at equilibrium length.

The damping constant at equilibrium length *during isometric contraction* is much higher than at rest and rises up to length 180 after which it decreases. Damping during contraction as function of tension is likewise higher than at rest. The damping constants for the contracting fibre *released* to the same tension as at rest coincide with the constants for the isometrically contracting fibre at the same tension.

Viscosity expressed as viscous stiffness (st_v = damp. constant $\times$ angular velocity) coincides as regards length and tension with the corresponding curves of the damping constants. st_v in proportion to elastic stiffness (st_e) at equilibrium length at rest and in isometric contraction is about 1 : 4, and at maximal length 1 : 15 and 1 : 10 at rest and in contraction respectively. Besides damping constant and viscous stiffness, *elastic after-effects* are a measure of viscosity in muscle. These are investigated by instantaneous elongations at rest and during contraction and are well marked in both cases. A closer analysis of the consolidation course can only be performed in resting muscle. At low elongations shortening during consolidation is 60—70 per cent of the initial increase in tension, at higher lengths attaining more than 90 per cent showing agreement with the proportion between dynamic and static stiffness. The consolidation course indicates that a great part of the viscosity is not distributed evenly in the muscle but lies in series with elastic elements. This is supported by experiments with different frequencies where the proportion $\frac{st_v}{st_e}$ decreases with increasing frequency.

The effect of *adrenalin* and *acetylcholine* on tension, stiffness and damping is investigated in the resting and contracting muscle. In the former no difference in elastic properties and viscosities is found between normal and drugged muscle. During contraction adrenalin causes a rise in tension, stiffness and damping when referred to the same length, but as function of tension, stiffness and damping coincide with that of normal fibres. Acetylcholine causes a corresponding decrease of tension, stiffness and viscosity. Here too there is no difference from normal muscle.

When comparing cardiac and skeletal muscle, contraction extratension attains its maximum much later in the former than in the latter. Yielding and locking are not found in cardiac muscle. The proportion of dynamic to static stiffness in cardiac muscle is many times that in skeletal muscle. Viscosity has a greater influence in cardiac than in skeletal muscle. The high viscosity in cardiac muscle can be of importance in cardiac function as it shortens contraction and contributes to the filling of the heart during diastole by facilitating a low diastolic initial pressure.

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References.

Blix, M., Skand. Arch. Physiol. 1892. 4. 399.

— Skand. Arch. Physiol. 1895. 5. 150.

— Skand. Arch. Physiol. 1895. 5. 173.

Bohnenkampf, H., and *Ernst, W.*, Z. Biol. 1926. 84. 436.

Bouckaert, J. P., Capellan, L. and *De Blende, J.*, J. Physiol. 1930. 69. 473.

Brown, G. L., Dale, H. H. and *Feldberg, W.*, J. Physiol. 1936. 87. 394.

Buchthal, F., D. Kgl. Danske Vidensk. Selskab. Biol. Medd. XIV. 2. 1942.

— *Kaiser, E.* and *Knappeis, G. G.*, Acta Physiol. Scand. 1944 in press.

— — Acta Physiol. Scand. 1944 in press.

— *Knappeis, G. G.* and *Lindhard, J.*, Skand. Arch. Physiol. 1936. 73. 163.

— and *Lindhard, J.*, D. Kgl. Danske Vidensk. Selskab. Biol. Medd. XIV. 6. 1939.

Dreser, H., Arch. f. exper. Path. 1888. 24. 221.

Ducret, S., Pflügers Arch. Physiol. 1931. 227. 81.

Eismayer, G., and *Quincke, H.*, Arch. f. exper. Path. 1929. 139. 313.

Fick, A., Mechanische Arbeit und Wärmeentwicklung bei der Muskeltätigkeit. Leipzig, 1882.

Frank, O., Sitzgsber. Ges. Morph. u. Physiol. Münch. 1898. 14. 147.

— Z. f. Biologie 1895. 32. 320.

Gasser, H. S. and *Hill, A. V.*, Proc. Roy. Soc. B. 1924. 96. 398.

Gompertz, C., Arch. f. Anat. & Physiol., Physiol. Abt. 1884. 242.

v. *Kries, J.*, Arch. f. Anat. & Physiol., Physiol. Abt. 1880. 348.

— Arch. f. Anat. & Physiol., Physiol. Abt. 1892. 1.

Levin, A. and *Wyman, J.*, Proc. Roy. Soc. 1927. 101. 218.

Meyer, K. H., Die hochpolymeren Verbindungen. Leipzig, 1940.

Sacerdote, E. P., J. de phys. 1898. 7. 516.

Sichel, F. J. M., J. cell. and comp. Physiol. 1934. 1935. 5. 21.

Triepel, H., Einführung in die physikalische Anatomie Weisbaden 1902. 1. 102.

— Tabulæ biolog. 1925. 1. 35.

Weber, E., Wagners Handwörterbuch der Naturwissensch. 1846. 3. 1—122.

Weber, H. H., Ergebn. Physiol. 1934. 36. 109.

Wöhlisch, E. and *Clamann, H. G.*, Pflügers Arch. Physiol. 1936. 237. 590.

— and *du Mesnil de Rochemont, R.* and *Gerschler, H.*, Z. Biol. 1927. 85. 325.

— Die Naturwissensch. 1940. 28. 305.

Wundt, W., Die Lehre von der Muskelbewegung. Braunschweig, 1858.

Zakarias, L., Tungsram Technische Mitteilungen. 1938. August. p. 103.

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BY

TAGE ASTRUP

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København, den 20. April 1944.

Bengt Strömgren,
h. a. dec.

TILEGNET

ESBJERG

MIN BARNDOMS BY
VED VESTERHAVET

"When I have the honor of being consulted by a young man who has not yet found himself intellectually but who is filled with the desire to devote his life to some branch of medicine, be it clinical medicine, pathology, hygiene, bacteriology, physiology or pharmacology, my advice always is, "Study chemistry at least three years. Try with all your power to master enough of this great science to start you on your career.""

JOHN J. ABEL.
(1857–1938)

(Used by R. A. GORTNER as an introduction to his "Outlines of Biochemistry".)

From *"Experimental and chemical studies of the blood with an appeal for more extended chemical training for the biological and medical investigator"*. SCIENCE *42*, 135, 165 (1915).

PREFACE

The present studies in the biochemistry of blood coagulation were carried out at the *Biological Institute of the Carlsberg Foundation*, Copenhagen, where I serve as Assistant in Biochemistry.

To Dr. med. ALBERT FISCHER, chief of the institute, whose work on the coagulation of blood I have continued, goes the credit for not only giving me the most excellent working conditions, but it was Dr. Fischer's inspiration that made me follow my inclination for scientific work. For the quite unique interest Dr. Fischer thus has taken in me and my work I owe him my most sincere gratitude.

I am greatly indebted to the *Carlsberg Foundation* for placing me in a position where scientific investigation can be carried out unhampered by other duties.

Most of the new experimental results published in this work are based on measurements carried out with great skill by Miss BIRGIT BRODSGAARD. In preparing the manuscript Miss INGER KIRK PEDERSEN has been of valuable help to me. Mr. JØRGEN ASTRUP has made the graphs and HANS ANDERSEN, M. D., has gone through my manuscript.

For grants during the work I express my thanks to "*Danmarks tekniske Højskoles Fond for teknisk Kemi*", and I am also obliged to "*Løvens kemiske Fabrik*" for assistance afforded to me on various occasions.

Finally I wish to acknowledge my debt to my former teacher in organic chemistry, Professor, Dr. phil. HAKON LUND, for his interest in me during my studies and later on.

Copenhagen, October 1943.

TAGE ASTRUP.

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INTRODUCTION

The author's studies on the coagulation of blood plasma form a continuation of the investigations performed by Dr. ALBERT FISCHER on this subject.

Dr. FISCHER's investigations were carried out under the impression of the widely divergent opinions of previous authors, as presented, for instance, in the reviews by OPPENHEIMER (*124*) (1926), WÖHLISCH (*172*) (1929) and HOWELL (95) (1935).

According to FISCHER (*73*) (1935), the blood coagulation may be looked upon as a specific case of the protein denaturation, according to which conception thrombin acts as a denaturating enzyme. He is here in accordance with the opinion expressed by WÖHLISCH (*174*) (1936). On the whole, the views of FISCHER (*71*) (1934) are founded on the so-called "classical theory of blood coagulation", but he is aware of the inadequacy of this theory to explain all the facts observed during investigation of the clotting process. In particular, the action of inhibitory substances, especially that of heparin, is not satisfactorily explained by the classical theory, neither in its original form nor in its modification by HOWELL. The theory therefore has to be extended on this point, cf. FISCHER (*73*) and FISCHER (*77*). The same is the case with the so-called autocatalytic proceeding of the blood clotting, which also is not explained by the classical theory (FISCHER (*76*)).

Already in the work of FISCHER the evaluation of the results obtained experimentally was made difficult by the confusion of the results of earlier authors and by their most contradictory opinions regarding the reaction mechanism. This drawback was felt even more during my own earlier investigations which were reviewed previously (*10*) (1938) and (*12*) (1939). The only way of overcoming this difficulty was to investigate all the fundamental reactions of the clotting process, using the

isolated reaction components in as pure a state as practically possible. For this purpose it was necessary first to work out methods for the preparation and purification of the substances participating in the coagulation process. Already during the work of FISCHER some investigations in this direction were begun especially concerning heparin (SCHMITZ & FISCHER (*141*)), fibrinogen (SCHMITZ (*140*)) and thrombokinase (FISCHER & HECHT (*81*)). It was decided therefore to proceed along this line: first try to isolate and investigate every single component of the coagulation process, and then investigate the interaction between such isolated and purified components. It was felt that only in doing so a reliable foundation for the understanding of the blood coagulation might be laid down. In this way investigations have been carried out concerning heparin, thrombokinase (thromboplastin), fibrinogen, thrombin, antithrombin and prothrombin. Some of the results obtained so far have already been published in different journals. Also some reviews of the results have been given, see for instance (*11*) (1938), (*16*) (1941), (*19*) (1942) and (*18*) (1942).

It is not the purpose of the studies presented in the following to give a complete review of the blood coagulation in the light of recent investigations by various authors, but only to extend and correlate the investigations carried out until now according to the above-mentioned plan.

Excellent reviews of the blood clotting have been given in 1929 and 1940 by WÖHLISCH (*172, 173*). Therefore only a brief chapter concerning the historical aspect of the clotting will be given. This will be followed by a chapter bringing some studies concerning the activation of the clotting process, namely the formation of thrombin. The next chapter will deal with the action of thrombin on fibrinogen, and the last chapter contains studies on the inhibition of the clotting by different substances.

Chapter I.

BRIEF REVIEW OF THE BLOOD COAGULATION

The difficulties in investigating and understanding the blood coagulation are of no new date. As early as 1899 O. HAMMARSTEN (*89*) said: "Die Lehre von der Faserstoffgerinnung hat leider schon gar zu viele unrichtige oder unbewiesene Theorien aufzuweisen, und der Grund hierzu liegt zum grossen Teil darin, dass man aus richtigen Beobachtungen seine Schlussfolgerungen oft zu rasch und ohne genügende Vorsicht gezogen hat."

The excellent comprehensive reviews by MORAWITZ (*122*) in 1905, WÖHLISCH (*172, 173*) in 1929 and 1940 show that this statement by HAMMARSTEN in all the following years by no means lost its meaning, but they show also that the warning given by HAMMARSTEN was only to a small extent followed by the later authors.

At a very early date the clotting of blood had drawn the attention of scientists. But not till the end of the 19th century did a real understanding of the complicated mechanism develop, and only by passing through several mistakes it has been possible, now, to give a description of the blood coagulation which is founded on a reliable interpretation of the experimental facts.

It has not been an easy task for nature to build up a fluid transportation medium which is able to circulate under a considerable pressure in a closed system, and which possesses the property of automatically repairing a possible leakage of the system produced by injuring the surrounding tissue. The solution of this problem is now known to be no simple one, and the circulating blood in an organism is in this respect a very complicated system in which several different components interact in a balanced manner in order to reach this object.

A very long evolution lies behind the coagulation process as presented by the blood of the higher organisms, and comparative studies have revealed a very interesting development from the simple agglutination of blood cells in primitive organisms to the elaborate combination between agglutination and fibrin formation in higher organisms. Our knowledge of this evolution is almost exclusively due to investigations carried out by LEO LOEB and his co-workers. It has been reviewed recently by SILBERBERG (*145*) (1938) in a paper on the causes and mechanisms of thrombosis. He summarises this section as follows: "These comparative investigations have shown then, that during the phylogenetic evolution of animals two processes developed side by side by means of which the organism reacted against interferences with the various constituents of the blood or body fluids, namely: *1*) an agglutination of cellular elements and *2*) a conversion of a colloid constituent of plasma, the fibrinogen, into coagulated fibrin. The first one of these two processes developed earliest; it is the only one existing in the more primitive organisms; in the higher organisms there was added to this the second one, the coagulation process. But even in the highest organisms the first, the agglutination process, persists and precedes coagulation. In invertebrates it is the amoebocytes, in the lower vertebrates the spindle cells and in mammals the bloodplates which primarily react by means of agglutination, and all these cellular elements show a similar mode of reaction to certain environmental changes. These two processes, agglutination and coagulation, therefore represent coordinated processes, and while in lower organisms the former is present alone or is much predominating, in the higher classes of animals the coagulation process becomes more and more accentuated."—

It has been a difficult task to investigate and elucidate such a complicated clotting system and to reach a fairly reliable conception of the underlying reactions. Our modern conception of the blood coagulation has been reached by passing through three stages.

First Period.

After the discovery of *fibrinogen* by DENIS (*59*) (1859) (cfr. also the investigation carried out by PANUM (*127*) in 1851), the first period ended in 1905 with the formulation of the so-called "classical scheme" for the blood coagulation. This period has been excellently reviewed by MORAWITZ (*122*) in 1905.

According to this "classical scheme" the coagulation proceeds in two phases as follows:

First phase: Prothrombin + thrombokinase + Ca^{++} → thrombin.
Second phase: Thrombin + fibrinogen → fibrin.

This conception is based on the discovery by ALEXANDER SCHMIDT of the coagulant substance *thrombin*, which is shown later to be of enzymatic character. Thrombin acts by converting the soluble protein *fibrinogen*, which is present in the blood plasma, into the insoluble *fibrin*. This reaction has especially been investigated in detail by O. HAMMARSTEN who has shown definitely that the presence of calcium ions is unnecessary for this part of the reaction.

That calcium salts must be present for the coagulation as a whole was first shown by ARTHUS & PAGES, who thus laid the foundation of the understanding of the first phase of the reaction, which was then formulated as described by MORAWITZ and FULD & SPIRO. According to these authors, thrombin is formed from an inactive precursor: *prothrombin*, present in blood plasma, by means of an activating substance called *thrombokinase*, resulting from the injured tissues or blood cells. Thrombokinase in conjunction with *calcium ions* converts prothrombin into thrombin.

Second Period.

When it is born in mind that the classical scheme for the coagulation was set forth as early as in 1904, and that it still forms the foundation of our understanding of the mechanism of clotting, one might expect that in the following time this scheme might have been definitely established and undergone further development. This, however, has been the case only

to a small extent, as it has taken about thirty years merely to obtain a general acceptance of the reactions as presented in the classical scheme.

The first difficulty was to determine the nature of the clotting-inducing tissue factor, in the preceding called thrombokinase, by others called cytozym and by most American authors designated thromboplastin. Some investigators found this activating substance in aqueous extract of tissues, while others found it in alcoholic extracts. The first group was of the opinion that it was a thermolabile substance. The latter group assumed it to be a thermostable lipoidal substance resembling cephaline. In 1912, however, it was possible to come to an agreement between these two groups, as it was established that the active substance consisted of an active lipoidal component bound to a protein (BORDET, HOWELL, ZAK).

An important delay was due, however, to the circumstance that the classical scheme was not completely accepted by the American authors, who until about 1935 founded their investigations on the two different theories proposed by HOWELL or MILLS. Of these two theories HOWELL'S may be looked upon as a modification of the classical theory, as he operates with the same substances, and his theory differs from the classical only in explaining the formation of thrombin.

The most important feature of HOWELL'S theory is the rôle he ascribes to the coagulation-inhibiting substances. These substances, as will be seen from the scheme, are not directly included in the classical theory; still, they are by no means in contradiction hereto, and already MORAWITZ himself was aware of the existence of such substances, but took them to be of minor importance only. HOWELL, however, is of the opinion that these substances play the principal rôle in the clotting process and assumes that prothrombin coupled with an inhibitory substance is present in plasma in a stabilised form. The tissue factor combines with the inhibitory substance and releases prothrombin in a free state, which under the action of calcium ions alone, undergoes activation to thrombin.

HOWELL'S theory was greatly supported when he succeeded in isolating the coagulation-inhibiting substance *heparin* in a

very active state. In keeping with his theory, he therefore assumes that the fluid state of the blood in the organism is due to the combination of prothrombin with heparin, while the absence of intravascular coagulation after the classical theory solely is due to lack of thrombokinase.

It must be pointed out, however, that the coagulation-inhibiting substances may be included in the classical scheme by only minor modifications. The classical scheme is concerned chiefly with dividing the processes into two phases, and the characterization of the reacting components, but speaks only little about the mechanism by which the interaction proceeds. MORAWITZ himself, however, was of the opinion that the tissue factor was an activating substance in its proper sense, wherefore he termed it *thrombokinase*.

The theory of MILLS was quite different from the classical theory, as he resumes an older theory, according to which the tissue factor, which he terms "tissue fibrinogen", reacts directly with fibrinogen to form fibrin.

For European authors the conditions were still more confused. As WÖHLISCH points out, the number of theories for the blood coagulation here exceeded the number of workers in the field, as some of the authors in the course of their investigations changed their views several times.

In Europe, in this period, especially J. BORDET, J. MELLANBY and WÖHLISCH based their works on the classical conception. Some of the other authors attached a chief importance to inhibitory substances, but most investigators presented their own new hypothesis, throwing the whole situation into a most serious confusion. This condition is clearly reflected in the reviews by OPPENHEIMER (*124*) (1926) and WÖHLISCH (*172*) (1929).

This confusion was due chiefly to the introduction of physical chemistry and colloid chemistry into the study of blood clotting. This, unfortunately, was done chiefly by workers who were not sufficiently familiar with the fundamental principles of these scientific branches, and who therefore made erroneous use of these principles, drawing premature or wrong conclusions. Instead of using physical chemistry and colloid chemistry to

obtain a deeper understanding of the mechanism of clotting, based on the conclusions already drawn from reliable experimental facts, most authors in this period used the new aspects simply to explain the whole blood clotting as a result of only one single transformation. Earlier investigations and the results of other authors were most often entirely neglected.

Contributing to this unfortunate development was undoubtedly the easy conversion of the liquid blood plasma (in the colloidal state of a sol) into the solid blood clot in the state of a gel. This transformation is apparently so easily performed and investigated that it seems possible for every one to draw his own conclusions about what is happening, which of course does no harm when not published as the results of scientific investigations. The purely colloidal phenomenon of a sol-gel transformation has so fascinated the observers that they forgot that for the reaction mechanism itself the character of the resulting end-product is of no importance. An enzyme reaction is, as is well known, not characterized by the physical properties of the end-products, but by the chemical transformations proceeding during the reaction.

Instead of bringing about a deeper understanding of the blood clotting, there resulted therefore a profound stagnation in this period with an abundance of the most contradictory theories regarding the clotting mechanism. Referring to this state of affairs HOWELL (*95*), as late as in 1935, says: "One seeks in vain to reconcile these views or to reduce them to a common basis."

Third Period.

At this juncture some American investigators published their studies, SMITH, WARNER & BRINKHOUS (*139*) in 1934, QUICK (*131*) and EAGLE (*62*) in 1935. They rejected the original ideas of MILLS and adopted the principles of the classical scheme as set forth by MORAWITZ and denied also the rôle ascribed to the inhibitory substances by HOWELL. Soon afterwards FERGUSON (*65*, *66*) also subscribed to the opinion of these authors.

The American investigations thus confirmed the conceptions accepted by the leading European workers, and the possibility

for a fruitful mutual influence was created. The further development, however, has to a great extent taken place in U. S. A., as the many years of confusion still hampers the work done in Europe. Thus it may be pointed out that OPPENHEIMER (*125*) (1939) and DYCKERHOFF (*60*) (1940) in their reviews on blood coagulation both are of the opinion that prothrombin is thrombin which is masked by combining with an inhibitory substance (heparin), a theory which shows some resemblance to the theory of HOWELL. This assumption seems not to be in accordance with reality—as pointed out, for instance, by WÖHLISCH (*173*) in his excellent review in 1940. WÖHLISCH here draws the conclusion that since his review in 1929, the confusion has changed into a great deal of elucidation, and that it is now possible to base future investigations on a reliable foundation.

According to the classical theory of blood coagulation as presented in the preceding we have to assume the following to happen when blood escapes from an injured blood vessel:

From the injured blood cells, especially the platelets, and the surrounding tissue cells thrombokinase (thromboplastin) is released. By a reaction not fully known, prothrombin present in the blood plasma is now transformed to thrombin by the cooperation of calcium ions. Thrombin then converts the soluble fibrinogen into fibrin, which in the presence of the blood proteins forms a sticky clot and thus together with the agglutinating platelets closes the leakage when the blood stream is not too vigorous.

Unexpectedly the study of blood coagulation has received a great stimulus from the vitamin research. The discovery by HENRIK DAM (see *55*) of vitamin K and the inability of the animal organism to build prothrombin without the presence of this vitamin aroused great interest and placed the blood coagulation in the middle of events, as it was now not only of scientific interest to investigate the clotting mechanism, but the results could find direct practical use in the clinic.

The rapidity with which shed blood clots is dependent on the velocity of thrombin formation, which again is dependent on the concentration of prothrombin in the plasma and the amount

of thrombokinase (thromboplastin) released from the cells. In case of decreased prothrombin concentration, for instance in vitamin K deficiency and in liver injury, less thrombin will be formed, and the formation will proceed more slowly. A bleeding tendency will thus develop for the organism in question. The determination of the prothrombin content of the blood is therefore of great importance, and since the discovery of vitamin K and its mode of action, the interest has centered about this question, and a large part of the papers published in recent years on blood clotting have been concerned with such problems.

Already HOWELL (*94*) (1914) has tried to give a measure for the prothrombin content by determining the clotting time after the addition of the optimal amount of calcium chloride to oxalated blood plasma. The time measured he called the "prothrombin time", as according to his theory, calcium ions alone were necessary for the activation of prothrombin. He pays no regard to the varying content of thrombokinase, and his method therefore yields unsatisfactory results. It was QUICK (*136*, *131*) (1935) who later solved this problem by adding a surplus of thrombokinase. By his method a measure for the prothrombin content is obtained by comparison with a diluted normal plasma. The method is highly suitable for clinical purposes, as it is easily carried out, but accurate determinations are not obtained as, for one thing, the presence of inhibitory substances is not taken into consideration.

Later many other methods for determination of the prothrombin content of the blood have been developed, but only three of these are based on new principles, while all the rest are modifications of these four methods. The three new methods are the following:

After the method of WARNER, BRINKHOUS & SMITH (*166*) (1936) prothrombin is first converted into thrombin, and the amount of thrombin formed is then determined by addition of a fibrinogen solution. After this method it is thus possible to investigate the two phases of the coagulation separately and to be sure that all the prothrombin is converted before the clotting takes place. The method is theoretically the most

satisfactory, but regard to the presence of inhibitory substance is paid only by diluting the plasma during the measurements.

In the method of DAM & GLAVIND (*56*) (1938) heparin plasma is brought to clot in a definite time by the addition of thrombokinase. The amount of thrombokinase added is used as a measure for the prothrombin content, because the amount of thrombokinase must be increased as the prothrombin content is decreased.

THORDARSON (*153*) (1940) tries in his method to combine the convenience of QUICK'S method with the more exact method of WARNER, BRINKHOUS & SMITH by using very dilute plasma solutions to which fibrinogen, thrombokinase and calcium chloride are added. With this method he has succeeded in demonstrating an increase of prothrombin in blood during pregnancy (*152*).

Details of the different methods and their modifications are given in the dissertation of THORDARSON (*154*) (1941) and in a paper by PLUM & LARSEN (*130*) (1941).

While thus the immediate results of the discovery of vitamin K especially have concerned the measurement and investigation of prothrombin, also other fields of the blood coagulation have been investigated. Here mention is to be made of the studies on conversion of fibrinogen to fibrin by WÖHLISCH and co-workers. These investigations are reviewed thoroughly by WÖHLISCH (*173*) in 1940. In spite of numerous experiments it has not yet been possible to explain what is happening in this reaction. As mentioned already, WÖHLISCH and FISCHER both assume the process to be a special case of a protein denaturation. But as the denaturation of proteins still forms an unsolved problem in biochemistry it is not possible from this to draw any definite conclusion about the fibrinogen-fibrin transformation.

Another question has also aroused great interest, namely the investigation of heparin and its actions. This interest is due to the probability of using heparin in practice as a prophylactic and therapeutic agent against thrombosis. The most important contributions have been made by JORPES and co-workers. These questions together with the other problems will be

discussed in the following chapters, however, as they concern my own investigations.

Although the classical theory of blood coagulation forms a solid foundation for the understanding of the mechanism of clotting, it is nevertheless very imperfect and needs further development. Thus it says nothing about the ways in which the different components react with each other and the properties of the substances taking part in the reaction. For instance, nothing is said about what happens when prothrombin is converted into thrombin, and very different opinions have therefore been expressed by various authors concerning this question. Such problems may be solved only by attempts to isolate and purify every single component and study its properties and reactions in the purified state. This principle has therefore been determining for the investigations to be presented in the following.

Also on other points the classical scheme must be extended. It has been mentioned already that the inhibitory substances must be incorporated in it. Further other reactions during the coagulation must find a place in the scheme too. This applies to the so-called autocatalytic reaction during the clotting. It is especially HOWELL and FISCHER who have reported investigations in these directions and pointed out the inability of the classical theory to explain all the experimental facts observed. Studies concerning these problems will also be mentioned in the following chapters.

Chapter II.

ACTIVATION OF THE COAGULATION

Here "activation of the coagulation" means the sequence of reactions which ends in the conversion of the precursor prothrombin into the active blood-clotting enzyme thrombin.

First will be discussed the properties and the action of the activating substance termed thrombokinase or thromboplastin, released from the platelets or the injured tissue cells. Then the properties of prothrombin and its transformation to thrombin will be dealt with. Further, the problems concerning the autocatalytic reaction during blood clotting are taken into consideration.

A. Properties and Action of Thrombokinase.

In our studies on the tissue factor we have confirmed the experiences of earlier authors, according to which this substance is very unstable and cannot be purified by means of the usual methods (see for inst. ASTRUP & DARLING (*24*)).

It is now known that thrombokinase consists of a lipoidal substance coupled to a protein, and in this manner it is possible to explain many of its properties, e.g., its solubility in aqueous solution and the presence in alcoholic extracts of an active lipoidal substance. The protein moiety has not been investigated to any extent, but the investigations of MILLS (*120*), MILLS & GUEST (*121*) and FISCHER (*72*) concern also partially the protein component of the tissue factor isolated either from ox lung or from chicken muscle. The purification of the tissue factor and its splitting into a protein and a lipoid part was also studied by FISCHER (*70*). Later FISCHER & HECHT (*81*) investigated the lipoidal substance alone (this time isolated

from pig brain) and, contrary to the investigations of HOWELL and his pupils and most other workers, they found that it is not identical with cephalin, as on purification its activity is decreased. This was later confirmed by CHARLES, FISHER & SCOTT (*51*) and recently the same conclusion was drawn by WIDENBAUER & REICHEL (*169*). The properties of the tissue factor isolated from different tissues (chicken) were also investigated by FISCHER (*75*) and lung tissue was found to give the most potent extract. Later such an extract of chicken lung was investigated by electrophoresis, FISCHER & HERRMANN (*82*).

A great many investigations on the tissue factor have been carried out by various authors, but the results have so far not been very satisfactory, as the substance has resisted any thorough treatment (cf. ASTRUP & DARLING (*24*)).

The best known of its properties is the relation between the amount of tissue factor and the clotting time. Thus FULD (*84*) (1902) and FISCHER (*74*) (1935) investigated the clotting of avian plasma by the addition of avian thrombokinase, and for this relation FISCHER found the following equation (1):

$$\frac{1}{t} = k \cdot c^a \qquad (1)$$

in which t is the clotting time, c the amount of thrombokinase added, and k and a constants.

This relation was already found by BARRATT (*41*) (1934) and KUGELMASS (*105*) (1923) to be valid for the clotting of citrated and oxalated mammalian plasma with thrombin, and MILLS (*120*) (1921) had found the same equation for the clotting of recalcified, oxalated plasma by addition of the tissue factor. The equation thus has a wide range of validity for the blood coagulation, but it must be mentioned that it is purely empirical and says nothing about the reaction mechanism.[1])

It is often mentioned that the law of SCHÜTZ concerning enzymatic action is valid for the blood coagulation, but, as

[1]) In an interesting paper just received R. G. LEGLER (Helv. Chim. Acta *26*, 1512 (1943)) discusses the kinetics of blood clotting, with special reference to the equation mentioned above.

has already been pointed out (ASTRUP (*9*) (1938)), this is due to a misunderstanding of Schütz's law, which is also found in reviews on this subject, namely HAMMARSTEN (*90*) (1926), WÖHLISCH (*171*) (1929) and OPPENHEIMER (*126*) (1939).

It was FULD (*84*) (1902) (cf. STROMBERG (*150*) (1911) and TSUNOO (*157*) (1925)) who first applied Schütz's law to the clotting of blood plasma. The question has later been treated in detail (ASTRUP (*8*) (1938)), and it is here pointed out that the original results obtained by SCHÜTZ (*142*) (1885) show that the amount of substance $[P]$ (albumin) converted in a given time is proportional to the square root of the amount of enzyme $[E]$ added. SCHÜTZ's law may therefore be written as follows (2):

$$[P] = \text{const.} \cdot \sqrt{[E]} \qquad (2).$$

In this equation the reaction time does not appear as a variable and so SCHÜTZ's law cannot, of course, be used for the characterization of time relations.

In blood clotting however, the reaction time t and the amount of active substance $[E]$ added are the only measurable quantities, while the degree of reaction $[P]$ is constant, and under such circumstances the original law of SCHÜTZ therefore is of no value.

Equation (2) may be extended in the following way (equation (3)):

$$[P] = \text{const.} \cdot \sqrt{[E]} \cdot f(t) \qquad (3)$$

where $f(t)$ expresses the unknown dependency of $[P]$ on the reaction time t. When the reaction time is constant, $f(t)$ is also constant, and the original law of SCHÜTZ is established again.

In the extended equation (3) the amount of enzyme $[E]$ (i.e. active substance) and the reaction time t depend in an unknown manner on each other. It is therefore necessary to know the relation between the amount of substrate converted $[P]$ and the reaction time t. This relation cannot be calculated but must be found by experiments. On the basis of experiments by SJÖQVIST (*146*) (1895) on pepsin digestion, ARRHENIUS (*3*) (1907) determined this relation and extended SCHÜTZ's law in

the following manner (4) which was also found by SCHÜTZ and HUPPERT (*143*) (1900).

$$[P] = \text{const.} \cdot \sqrt{[E] \cdot t} \qquad (4).$$

Equation (4) only expresses reciprocity between $[E]$ and t and is not the law used by FULD. The rule referred to by FULD as SCHÜTZ's law can be formulated as follows (5):

$$\frac{1}{t} = \text{const.} \cdot \sqrt{[E]} \qquad (5).$$

On comparing equation (5) with equation (2) it appears evident that an incorrect use of $[P]$ and $\frac{1}{t}$ as interchangeable measures of reaction velocity has led to this misunderstanding of SCHÜTZ's law. Later, however, KUGELMASS (*105*) (1923) used the extended equation (4) and discussed the reciprocity between the clotting time and the amount of active substance.

In enzyme chemistry it is very common to use the amount of substrate converted as an arbitrary measure of reaction velocity, and the same applies to the reciprocal of the time used for a given reaction. The experimental conditions decide which alternative will be chosen in a given situation. When the relation between the reaction time and the amount of substrate converted is not known, however, it is not permissible to use these measures as expressions for the reaction velocity of an enzymatic or chemical reaction, as only under certain circumstances the conditions for this will be satisfied. The question is dealt with especially by BODANSKY (*44*) (1937) who shows that a general confusion exists on this point in enzyme chemistry.

Various other formulas have been tried by different authors (see ASTRUP (*8*)), but only equation (1) can be used to any greater extent. For practical purposes it is easier to use it in the following logarithmic form where $b = log\ k$:

$$log\ t = -a\ log\ c - b$$

or

$$log\ c = \frac{1}{a}(-log\ t - b) \qquad (6).$$

In this form the relation has been used by MILLS (*120*) (1921) for investigation of the properties of the tissue factor isolated from beef lung, and we have used it for the same purpose (ASTRUP & DARLING (*24*) (1942)). A condition for using equation (6) for determination of the potency of a thrombokinase solution is that the value of a is constant during the experiments. It has already been pointed out (ASTRUP (*8*)) that the value of a is characteristic of the reacting system, as it only depends on the experimental conditions—and neither on the amount of active substance added nor on the units used for the measurement of the clotting time or the concentration of active substance.

In our investigations we have confirmed the results of MILLS so far as they concern the applicability of the formula to the clotting of recalcified mammalian plasma by the addition of thrombokinase derived from lung. By plotting a graph with *log c* as abscissa and *log t* as ordinate, straight lines are obtained from which it is possible with reasonable accuracy to obtain a determination of the potency of the solution of thrombokinase used. Under the given experimental conditions the value of a was found for thrombokinase from beef lung and oxalated ox plasma to be 0.200.

When a unit of thrombokinase (K.U.) is set as the amount of active substance which under the given experimental conditions will clot 1 ml of recalcified oxalated ox plasma in 1 minute, b is -1.7782 (independent of a) and the following general equation (7) is obtained, by means of which it is possible to calculate the strength of the solution of thrombokinase used from the determination of the clotting time in seconds.

$$\log c = \frac{1}{a} \cdot (1.7782 - \log t) \qquad (7).$$

Due to the form of the equation it is only possible to make the estimations with a rather poor accuracy (see ASTRUP & DARLING (*24*)), but so far no better method is available. We have, for instance, used the equation for studying the inactivation by heating of a solution of thrombokinase from ox lung.

These studies have now been extended also to thrombokinase derived from ox brain.

While for investigations on prothrombin, thrombokinase from lung has been used by SMITH, WARNER & BRINKHOUS (*139, 166, 167*) and THORDARSON (*153, 154*), most other investigators have used a thrombokinase derived from brain as described by QUICK (*131*), as this factor seems to be more stable and more active than lung kinase. As stable and active preparations of thrombokinase are necessary for the clinical determination of the prothrombin content of blood, great interest has in recent years been paid in investigations on this substance, and many papers have appeared concerning its preparation and its properties, e.g. by PLUM and LARSEN (*130*).

In his investigations QUICK (*131*) shows that the shortest clotting time, the so-called "prothrombin time", is increased when the "activity" of the preparation is decreased. This indicates that the prothrombin time is not only dependent on the potency of the thrombokinase solution, but that some unknown properties of the solutions also must play a rôle in the clotting process. It follows that comparison of the strength of different solutions is impossible, while the properties of the preparations vary, not only in quantity but also in quality. QUICK uses the prothrombin time as a means for expressing the activity of his thromboplastin solutions, but he is aware of the problem and says: "The degree of activity must not be confused with thromboplastin content." In our investigations on thromboplastin from ox lung we found no indication of a qualitative difference in the action of the various preparations, as we always obtained curves corresponding to a value about 0.200 for the exponent a in equation (1). By using thromboplastin from brain it was found, however, that this exponent could vary considerably, thus showing qualitative differences in the properties of the different preparations. It is difficult to explain how this alteration in the reaction mechanism takes place.

Based on the principles described by QUICK (*131*) different methods were tried for the preparation of powerful products. The most active preparations were obtained by the following procedure:

Preparation of thromboplastin (Q-9).

A fresh ox brain is cleansed, blood and most of the membranes are removed. It is then passed once through a meat chopper and the mass is pressed through two layers of gauze. Of the pulp resulting, 100 g are added to 300 ml of salt solution (0.9 or 2.5 per cent NaCl) and 1 ml of 90 per cent phenol. The suspension is then treated 24—48 hours in a ball mill, giving a very fine suspension, which is used as such or diluted with physiological saline. It may be kept for several months in the ice-box without decreasing so much in activity that it has to be discarded.

Q-9.1: 10 ml of Q-9 is diluted to 25 ml and used as stock solution.

Q-9.2: 75 ml of Q-9 is dried in thin layers under evacuation with an oil pump. Yield 5.1 g dry substance. 1.0 g of Q-9.2 corresponds to 37 ml of Q-9.1.

Q-9.3: An amount of Q-9.2 is ground twice in a mortar with acetone. After filtering it is washed with acetone and dried in the air. The dry substance is again ground thoroughly in the mortar. By this treatment an amount of lipoidal products is removed (cholesterol) and the thromboplastin is obtained as a fine powder.

Dilutions are now made of these preparations and the clotting time measured in water-bath at 37° as described before (ASTRUP & DARLING (*24*)) by placing one ml of oxalated ox plasma in a tube containing 0.10 ml of the thromboplastin solution in question and an optimal amount of calcium chloride solution. The plasma must not have been stored (at 0°) longer than three days if sufficient accuracy is to be obtained. Of Q-9.2 and Q-9.3 an amount of the dry substance is extracted at 37° for half an hour with sufficient solution to give a suspension corresponding to the content of the original suspension, and then centrifuged for one minute. From the results, the following curves are plotted, Fig. 1, where c is expressed in per cent of the original solution.

From Fig. 1. it is seen that the points lie on straight lines with a sufficient accuracy, with the exception of the clotting times (about 19 seconds) obtained with the highest concentrations of Q-9.1. With such concentrations all prothombin is activated, giving the "prothrombin time", which here is reached with Q-9.1 but not with Q-9.2 and Q-9.3. The slope of the straight lines is almost the same in all three cases, and a is determined to be about 0.27 with a slight tendency to increase for the treated preparations. The strength of the suspension may therefore be determined from the equation (7).

Due to the differences in the values of *a* for thromboplastin from lung and from brain it is not possible to compare such different products, but it is only possible to compare the three curves with each other. It is seen that the potency of the suspensions is greatly decreased by drying (Q-9.2) and further decreased by acetone treatment of the dried product (Q-9.3). The concen-

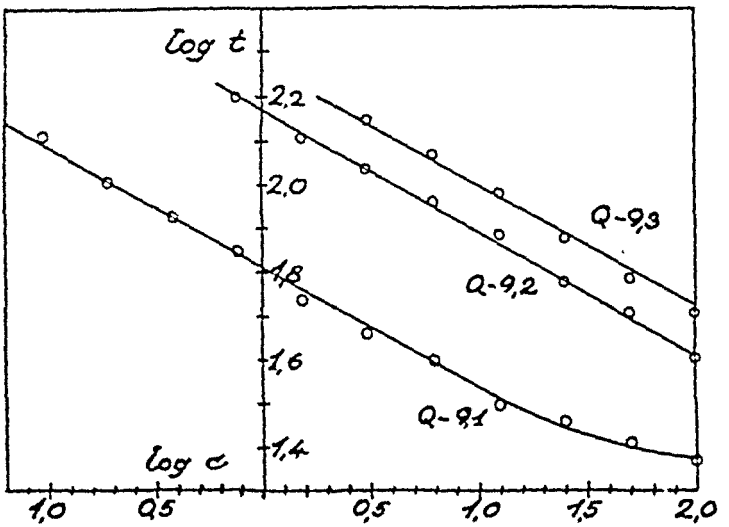

Fig. 1. Action of ox brain thrombokinase on recalcified oxalated plasma. Q-9.1, undried; Q-9.2, dried in vacuo; and Q-9.3, dried in vacuo and treated with acetone.

trated Q-9.3 gives a logarithm of the clotting time about 1.71 and corresponds to log c = 1.64 for Q-9.2 and log c = 0.40 for Q-9.1, where c is expressed in per cent. So the most concentrated suspension of Q-9.3 corresponds in strength to 44 per cent of Q-9.2 and only to 2.5 per cent of Q-9.1. By treating Q-9.1 as described, most of its potency is thus lost.

It has already been mentioned that the preparations of thrombokinase from brain are much more stable than the preparations from lung, and they also better stand treatment by various physical and chemical means (drying and acetone treatment for instance). This probably is due to a lower content of enzymes and mucins in the brain product.

A new lot (Q-14) of thrombokinase is now prepared as Q-9 and divided in two portions, of which one is left standing at

room temperature, and one is placed in the ice-box at 0°. After standing for three months they are compared with each other and with the original lot of Q-9, which is now eleven months old and most of the time has been standing at 0°.

Already in their appearances there are differences between the two lots of Q-14. The suspension of Q-14.2 which had been

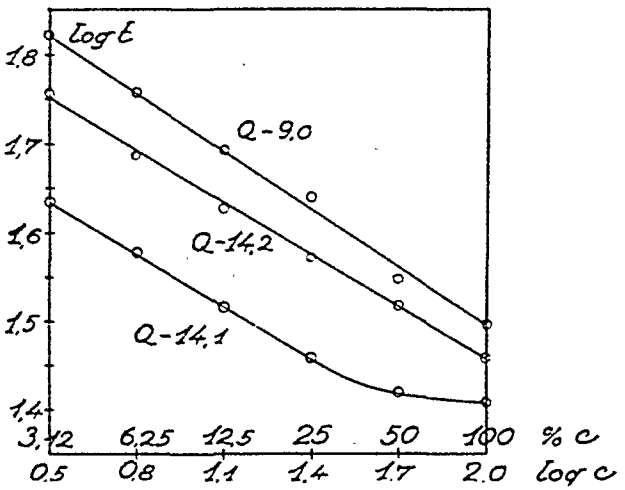

Fig. 2. Action of ox brain thrombokinase of different origin and under different treatment.

standing at room temperature is coarser and precipitates in a short time, while Q-14.1 is still finely dispersed. The clear liquid standing over the precipitate is only slightly colored in Q-14.1, while Q-14.2 shows a yellowish-brown color. Also the smell is different, and Q-14.2 smells a little putrefied. The measurements give the curves shown in Fig. 2. The value of a is about 0.20, and the three curves may therefore be compared with each other with sufficient accuracy. It is worth while to notice that the slope for Q-9 now is decreased as compared with the test just mentioned, which may be due to the new lot of plasma used.

From Fig. 2 it is seen that the concentrated Q-9 corresponds to $\log c = 1.79$ for Q-14.2 and $\log c = 1.19$ for Q-14.1, equal to about 62 per cent of the potency of Q-14.2 and 15 per cent of Q-14.1. Q-14.2 thus contains about 24 per cent of Q-14.1. It is further seen that by using Q-14.1 the prothrombin-time

of the plasma is found, although the suspension of thrombokinase is three months old. The curves show the great importance of the temperature to the stability of the preparations. The clear liquid obtained by centrifuging is practically inactive. In this connection the brain thrombokinase differs from lung thrombokinase and also from the suspensions made from dried

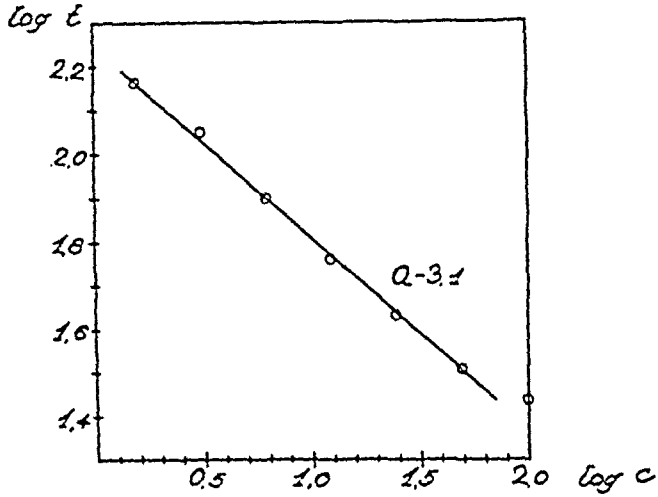

Fig. 3. Action of air-dried ox brain thrombokinase.

preparations, where the products obtained stand a short centrifuging.

It has already been mentioned that with brain thrombokinase the exponent *a* showed variations from one experiment to another. This was not the case with lung thrombokinase. By using suspensions of untreated brain (as in Q-9.1) the value of *a* was more equal to the value obtained with lung kinase, but by using dried preparations there was a greater variability, and as a rule *a* was considerably higher in those cases than usual. The variation thus seems to be dependent on the properties of the preparation, but also the plasma has shown its influence, and apparently to a greater extent against the dried preparations than against the preparations of untreated brain tissue. So probably the constancy of *a* found during our studies on lung kinase may be explained in part as due to favorable experimental conditions.

Examples of the variation of *a* are described in the following.

Q-3.1: Fresh ox brain is treated as described under the preparation of Q-9, and the pressed mass is dried in thin layers on vertically placed glass plates at about 35-40°, and finely ground in a mortar. One g is treated with 10 ml of physiological

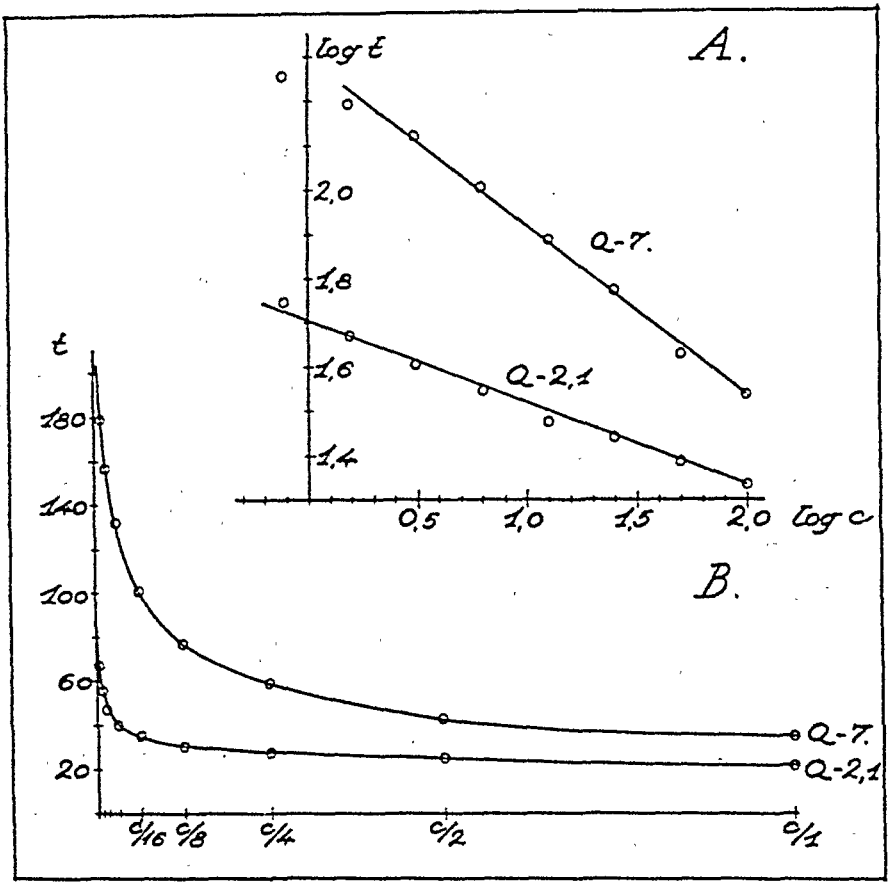

Fig. 4. Action of dried (Q-7) and undried (Q-2.1) ox brain thrombokinase. *A:* Logarithmic curves. *B:* Direct curves.

NaCl for half an hour at 37° and centrifuged for one minute. The solution gives the curve shown in Fig. 3, from which a is determined as 0.43. This is considerably higher than hitherto met with, which means that the dilution cannot be carried out so many times as usual during the measurements.

Extraction of Q-3.1 with boiling acetone (Q-4) does not alter the value of a, but the potency is decreased to less than half of the potency of Q-3.1, which in itself is considerably less

active than Q-9.1. On examination of the action of Q-3.1 and a preparation Q-2.1, prepared as Q-9.1, on the same plasma at the same time a is found for Q-3.1 to be 0.42 and for Q-2.1 to be 0.25. It is impossible to compare the activity of two such preparations, as dilution lowers the strength for Q-3.1 much more than the strength of Q-2.1 as measured by their respective clotting times. The cause for this qualitative difference in their action is still unknown.

Another example is shown in Fig. 4.

In this a sample of Q-2.1 is compared with the same product (Q-7) after drying in vacuo by room temperature in a desiccator over concentrated sulphuric acid and solid NaOH. The drying was not carried out so rapidly as in Q-9.2. As seen from Fig. 4, there results a considerable increase in the value of a contrary to what happened to Q-9.1 on drying. For Q-2.1 a is found to be about 0.18, which is lower than usual, for Q-7 a is 0.38. For comparison Fig. 4 also shows some curves, where c and t are plotted instead of $\log c$ and $\log t$. This gives the direct relation between c and t.

As drying is thus found usually to cause not only a decrease in activity of the product, but also to alter the value for a by increasing it, indicating that the dried product cannot stand the same dilutions as the undried product, it is best to use an undried preparation for investigations, where an active thrombokinase is to be used, for instance in the determination of the prothrombin content of blood. Of importance in this connection is also the fact that thrombokinase prepared from brain is practically free from prothrombin and therefore need not be heated to 60° for inactivation of prothrombin with a resulting considerable loss of potency. Our preparations could not clot solutions of prothrombin-free fibrinogen, prepared as described by ASTRUP & DARLING (*26*), by addition of calcium chloride.

Precipitation with acetone or drying decreases the activity of the brain thrombokinase, while the lung thrombokinase is almost completely destroyed by such treatment. Also direct treatment of the brain pulp with acetone after QUICK (*135*) gives some inactivation and a higher value for a, namely for Q-13 $a = 0.30$, resulting in a limitation of the dilution interval.

Treatment with alkali and acid inactivates the thrombokinase. Table I shows an example of such an inactivation.

Table I.

To samples of one ml of Q-2.1 are added varying amounts of 0.2-n NaOH, and after standing for five minutes at room temperature the corresponding amount of HCl is added; then the volume is adjusted to 2 ml with water. The clotting times are determined as usual. The amount of thrombokinase units (K.U.) per ml is then calculated, using equation (7) and putting $a = 0.20$.

NaOH ml	t mean	log t	log c	K.U. per ml
0.0	23.9	1.3784	1.999	1000
0.1	27.1	1.4330	1.726	530
0.2	29.5	1.4698	1.542	348
0.3	37.8	1.5775	1.004	101
0.4	48.4	1.6849	0.467	29
0.5	56.8	1.7544	0.119	13

From Table I it is seen that the inactivation proceeds very rapidly with only relatively small amounts of sodium hydroxyde. The same is the case on employment of acid. The pH obtained was not measured.

The influence of heating was also investigated in the same manner as was done with lung thrombokinase by ASTRUP & DARLING (*24*). Samples of thrombokinase were heated in a water-bath at different temperatures for a varying length of time.

Fig. 5 shows some results obtained by using a preparation (Q-4) from dried and acetone-treated ox brain and by using a preparation (Q-9.1) of untreated brain. The curves are obtained by calculating the concentration by means of the clotting time as usual and by using $a = 0.33$ for Q-4 and $a = 0.20$ for Q-9.1. Contrary to the curves obtained previously with ox lung thrombokinase, no increase in the activity by heating is found. The two preparations seem to be influenced by heating about equally.

It may be mentioned that a difference in the qualitative action of treated and untreated thrombokinase has been described

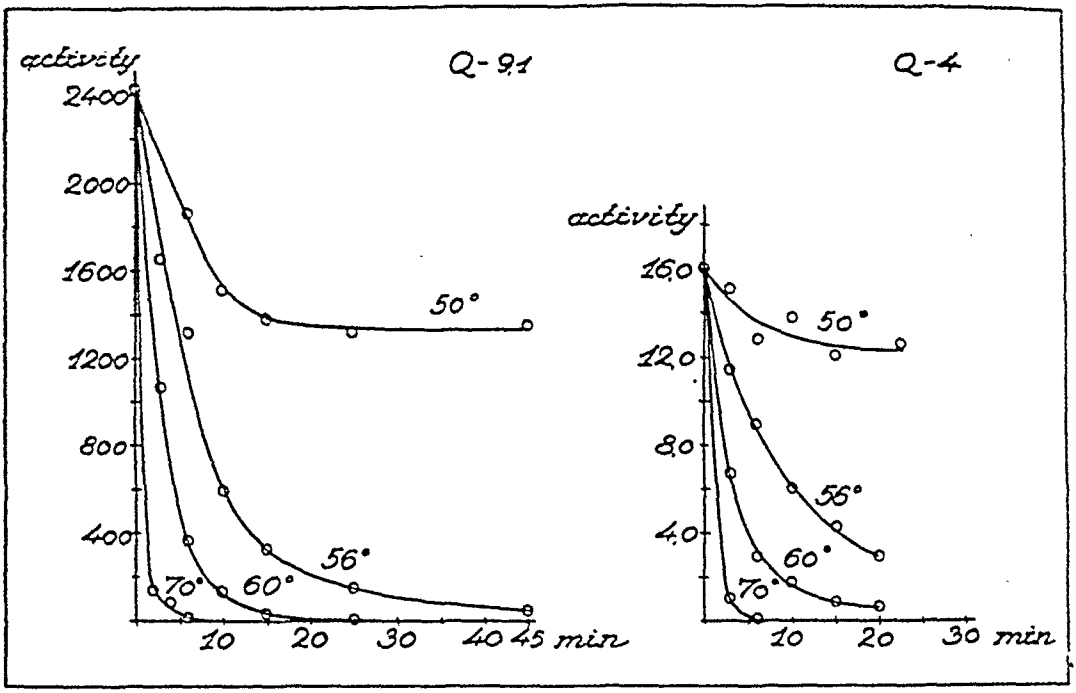

Fig. 5. Inactivation of ox brain thrombokinase by heating.

also by PLUM & LARSEN (*130*) (1941), namely, by determination of the prothrombin time.

B. Prothrombin and Its Conversion into Thrombin.

The conversion of prothrombin into thrombin has been the object of numerous investigations, and many views have been expressed concerning its mechanism (see the reviews given by WÖHLISCH (*172*, *173*)).

To solve this problem we have tried to isolate and purify prothrombin and thrombin in order to investigate their properties in a pure state. So far we have only succeeded in devising a simple method for the preparation of thrombin in a relatively pure state, while it has not yet been possible to find a satisfactory method for the preparation of purified prothrombin solutions. This seems to be due to a greater stability of thrombin and to some of its other properties, which make it easier to handle.

Thrombin was prepared by combining the methods of J. MELLANBY (*110*) (1909), (*112*) (1933) and BLEIBTREU (*43*) (1926)

and this work has been described in detail previously (ASTRUP & DARLING (*21*, *22* 1940, 1941)).

The method is based on the fact discovered by MELLANBY (*110*), that by addition of acid to a diluted blood plasma a fibrinogen is precipitated which contains a large amount of the prothrombin present in the plasma. By dissolving the precipitate and adding calcium chloride and thrombokinase the fibrinogen is transformed into fibrin, and a solution containing thrombin is formed. This solution is precipitated with acetone, and a crude thrombin containing about 800-1000 thrombin units (T.U.) per gram of substance is obtained. By treating this product with physiological saline and reprecipitation with acetone a preparation called *thrombin B* is obtained which contains about 10,000 T.U. per gram. A preparation of this kind was used for all our investigations where thrombin was used as a clotting agent—for instance in the investigation of antithrombin described in Chapter IV. Also its action *in vivo* has been studied, (ASTRUP & VOLKERT (*37*) (1943)) and it has been used as a hemostatic agent by SELSØ (*144*) (1943).

A thrombin unit was first defined as the amount of active substance which would clot 1 ml of oxalated ox plasma in 30 seconds at 37°, but later it was found that it was necessary to choose a standard preparation for comparison in order to avoid variations in the potency found, as the action was dependent on the conditions of the plasma used. Further it was found that a purified fibrinogen solution was more suitable for carrying out the measurements of thrombin activity, which have been described recently (ASTRUP & DARLING (*26*)).

The thrombin B could be further purified by ammonium sulphate precipitation and the active substance was found in the albumin fraction as it was not precipitated at half saturation. A still further purification was obtained by precipitation at pH 4.4 in salt-free solutions. This preparation was still impure and contained substances resulting from the thrombokinase added. By electrophoresis in the apparatus of TISELIUS (*155*) at pH 7.2 it showed two components, the fastest being the thrombin. Thrombin thus carries a large electrical charge

at neutral reaction which corresponds with its precipitability at pH 4.4.

The thrombin solution isolated by electrophoresis showed 1330 T.U. per mg of nitrogen which means that one mg N in the form of thrombin (with an N content of about 14 per cent) will clot 1330 ml of oxalated ox plasma in about 30 seconds. One mg of substance will thus clot about 200 ml plasma in 30 seconds, and in longer times it can clot even larger amounts of plasma. Plasma generally contains from 0.2 to 0.4 per cent of fibrinogen; correspondingly 200 ml plasma gives about 600 mg fibrin, *i.e.* about 600 times the amount of thrombin used for carrying out the conversion to fibrin in half a minute. It seems therefore unlikely that thrombin is not an enzyme, which has been assumed by several authors, while some have taken fibrin to be a chemical compound between thrombin and fibrinogen. But thrombin presents all the general properties of an enzyme. It thus acts in minute amounts in proportion to the substance acted upon. It is very thermolabile and is destroyed already by heating to 56° for 5 minutes. It may be partially recovered after the reaction when purified fibrinogen solutions are employed, since the fibrin formed adsorbs a part of it. In plasma, however, thrombin is inactivated due to the presence of antithrombic substances. Thrombin, therefore, must be considered an enzyme, and the conversion of fibrinogen to fibrin is an enzymatic reaction, namely an enzymatic, specific protein denaturation (Wöhlisch, Albert Fischer).

We have also tried to isolate prothrombin from the solution of Mellanby-fibrinogen before the addition of thrombokinase, but these experiments have not been as successful as the corresponding experiments on purification of thrombin. These studies were published recently (Astrup & Darling (*30*) (1943)). It was not possible by our method to prepare so stable and potent preparations as were prepared by Mellanby (*111*) (1930) and by Seegers, Smith, Warner & Brinkhous (*138*) (1938) using more elaborate methods (cf. also Seegers (*137*) (1940)). Our preparations were contaminated with proteolytic enzymes which caused inactivation of the prothrombin solutions in a few hours, and which it was impossible to get rid of by any of

the methods tried. The problem has already been discussed in detail in the paper mentioned.

By using ammonium sulphate precipitation, however, we succeeded in showing that prothrombin belongs to the globulins, which is in accordance with the findings of other authors (cf. CEKADA (*50*) (1926) and SCHMITZ (*140*) (1933)). This is very interesting as thrombin, according to our investigations, belongs to the albumins. We demonstrated this both by isolating prothrombin and thrombin in a purified state and examining the products by making ammonium sulphate precipitation curves. We further demonstrated the transformation directly by making ammonium sulphate precipitation curves for a prothrombin solution before and after activation by thrombokinase and calcium chloride (ASTRUP & DARLING (*28*)).

As it was not possible to show the presence of phosphorus in the purified thrombin preparations, this substance cannot contain a phosphor-lipid from the thrombokinase as an integrating part. Thrombin, therefore, cannot be a combination between prothrombin and thrombokinase—as set forth by several authors. While prothrombin did not pass through the dialysing membranes used (cellophane casings), thrombin slowly passed through and could be demonstrated by its clotting ability.

Its molecule therefore is a smaller one than the prothrombin molecule. This is in accordance with the properties of thrombin, as an albumin, and prothrombin as a globulin. According to the well-known investigations by SVEDBERG and his co-workers (see SVEDBERG & PEDERSEN (*151*)) with the ultracentrifuge, the globulins generally show a molecular weight about 150000, while the albumins as a rule have only half of this. It is therefore conceivable that the thrombin, far from being a prothrombin complex, is formed by a splitting of the prothrombin molecule by the influence of thrombokinase and calcium ions. The conversion may possibly be looked upon as a proteolytic splitting of an inactive globulin molecule (prothrombin) into one or more smaller active albumin molecules (thrombin). When more purified and stable prothrombin preparations have been made it will be possible to solve this problem definitely.

The function of thrombokinase in converting prothrombin to thrombin is still an unsolved problem, of which various views have been advanced by different authors. According to the original conception of MORAWITZ, it is a catalytically acting substance, which does not participate stoichiometrically in the reaction. This opinion has been supported by several authors —e.g. EAGLE (*62*) (1935)—but recent investigations by MERTZ, SEEGERS & SMITH (*115*) (1939) seem to show that there is a quantitative relationship between thrombokinase, prothrombin and the amount of thrombin formed. The fate of thrombokinase in this case is unknown, as it cannot form part of the thrombin molecule, as already pointed out. Our curves for the activation of prothrombin (ASTRUP & DARLING (*30*)), in which the prothrombin content was varied, indicate a reaction velocity dependent only on the concentration of prothrombin, which may be due to the thrombokinase acting as an enzyme or to its presence in a great excess.

On comparison of the curves obtained by PLUM & LARSEN (130) by varying the prothrombin content and our curves on the variation of thrombokinase (ASTRUP & DARLING (*24*)) the same relationship is found, and this makes it probable that prothrombin and thrombokinase may act as two components taking equal part in the activation process. The question, however, needs further investigation for its solution.

C. The Autocatalytic Reaction.

Several authors assume an autocatalytic reaction to take part in the clotting of blood, and some of the investigations concerning such a reaction have been discussed by WÖHLISCH (*173*) in the light of recent work on blood clotting. He arrives at the conclusion that an autocatalytic reaction may possibly proceed during the clotting, and that its character has not yet been demonstrated definitely. He further calls attention to the possibility of the proteolytic enzymes in blood and serum, which are known to be present in inactive form as proenzymes in blood, as probably playing a rôle in this process.

The accelerated formation of thrombin during the clotting

process was observed by several authors. First of all ARTHUS (*4, 5*) in 1901 made this observation, and soon BORDET & GENGOU (*45*) (1904) confirmed it. The last-mentioned authors also showed that the reaction could be repeated by transfer of a small fraction of the clotting mixture to fresh plasma, but it was FISCHER (*76*) who in 1935 showed that it was possible by using pure chicken plasma to repeat the process an infinite number of times and thus definitely showed that in the normal clotting of fresh plasma an autocatalytic reaction takes part. Many signs indicated that probably thrombin, formed through autocatalysis from prothrombin, was the cause of the autocatalytic process, and that thrombin thus corresponded to the two enzymes especially studied by NORTHROP (*123*) and his co-workers, pepsin and trypsin, which are formed autocatalytically from their precursors pepsinogen and trypsinogen.

A more thorough study of the question has shown the reaction to be more complicated, however, and the whole problem has therefore been treated in detail (ASTRUP (*15*)). Here it will be dealt with but briefly.

After the investigations by FISCHER (*76*), in which the acceleration of the clotting process was observed and its autocatalytic character realized, attempts were made to obtain curves for the increased formation of active substance (ASTRUP & FISCHER (*31*)). From these it is evident that an accelerated reaction takes place. Further investigation (ASTRUP (*8*) (1938)) on the relationship between the clotting time of chicken plasma and thrombokinase or thrombin brought forth the possibility that it was thrombin which was formed autocatalytically from prothrombin and thus was the cause of this reaction, and it was possible under this assumption to derive equations which qualitatively expressed the relations observed.

By subsequent investigation of each participating reaction separately it was found, however, that thrombin is *not* formed autocatalytically from its precursor prothrombin (ASTRUP (*15, 13*)). In the experiments it was impossible to carry the reaction further by using a prothrombin solution with or without the presence of calcium ions, although such a solution by addition of minute amounts of thrombokinase yielded large

amounts of thrombin through an accelerated reaction. Also in plasma from which prothrombin was removed by treatment with tricalcium phosphate, or in which its activation was hindered by removing the calcium ions, for instance, by addition of potassium oxalate, sodium citrate or sodium fluoride, it was impossible to carry the process further. The autocatalytic reaction proceeded only in fresh plasma, and it was hindered by addition of small amounts of acid.

While from these experiments it is quite clear that neither the formation of thrombin nor the conversion of fibrinogen into fibrin is responsible for the autocatalytic reaction, it still is an open question what substance is the cause of this. The curves found for the thrombin formation corresponded to the curves for the conversion of fibrinogen into fibrin as obtained by different physical means, e.g., light absorption. These two processes, however, have nothing to do with each other, and proceed as separate reactions side by side. The thrombin is formed without connection with the fibrin formation, and the transformation of fibrinogen begins as soon as the first amount of thrombin has been formed and proceeds with an increasing velocity, when the amount of thrombin is increased, but without any definite time relation to the thrombin formation, as they are separate processes. As the fibrin formation therefore is no direct measure for the thrombin formation, it cannot be used as a measure for this reaction, and the concordance of the two sets of curves is only incidental and due in part to the quite rapid reaction of fibrinogen in the presence of thrombin and to the rather low accuracy of the measurements of the thrombin formation. It was further shown that it was possible on the assumption of a catalytic formation of thrombin to calculate the conversion of prothrombin into thrombin, and that the curves so obtained corresponded to both the curves experimentally found for the thrombin formation and the fibrin formation. The curves therefore are only formally in agreement with the experimentally obtained results, and they allow of no conclusion as to the reaction mechanism. It was also shown that most of the earlier investigations concerning

the autocatalytic reaction were quite unsatisfactory in this respect and did not allow of any definite conclusions.

The autocatalytic reaction in fresh plasma must therefore be due to a still unknown process. Some findings indicate that it may be a conversion of a substance into an active thrombokinase which then activates prothrombin into thrombin. It seems to be well established that in plasma there occurs an activating substance in an inactive form, as a "prokinase" which by different means may undergo activation to a real thrombokinase (see the review by WÖHLISCH (*173*) and FEISSLY (*64*)). If this activation proceeds autocatalytically such a reaction will suffice to explain the observed facts. Some orientating experiments in this direction have been carried out, but as the substances in question seem to be very labile, a reliable method for their investigation has not yet been found.

For one thing the conception is supported by the results of the following experiments:

To 50 drops of normal chicken plasma are added 5 drops of a diluted chicken thrombokinase (an embryonic extract), so that the mixture clots in about 8 minutes at 37°. From such a clotting mixture 2 drops are transferred every minute to 5 drops of fresh plasma, and the clotting time of this new sample is determined. This experiment which is the same as performed by FISCHER (*76*) gives decreasing clotting times, indicating an increasing amount of an active or activating substance. An example is shown in Table II.

Table II.

Time for removal of sample in minutes	Clotting time after addition to fresh plasma in minutes
1	24
2	21
3	18
4	15
5	10
6	3
7	0.5
8	(clotting.)

From Table II it is quite clear that the amount of an active substance is rapidly increased during the clotting process. If this substance is thrombin, it will be possible, for instance, to bring about coagulation of oxalated plasma with samples removed from the mixture. By trying this, however, it was found quite impossible to bring oxalated chicken plasma to clot by addition of samples removed from a clotting plasma. Even the sample removed immediately before clotting, and which would clot fresh plasma in half a minute, showed no sign of coagulation in oxalated plasma in two hours. This is rather unexpected and shows decidedly that the active substance cannot be thrombin.

The clotting of the original mixture occurred, as already mentioned, in about 8 minutes. This point of the coagulation is determined by the conversion of fibrinogen into fibrin and is thus only indirectly dependent on the amount of thrombin present and its formation. In the previous experiments (*15*) it was shown that at the clotting point only a minimal amount of thrombin had been formed, and that far larger amounts still appeared after the coagulation of the mixture had occurred.

Obviously there is not sufficient thrombin, even in the sample removed just before clotting of the mixture, to bring about coagulation in the oxalated plasma, before the small amounts present have been inactivated completely by the powerful antithrombin contained in plasma and serum, and which by a relatively slow reaction in the course of several minutes is able to inactivate large amounts of thrombin (see the curve before, ASTRUP (*15*) as Fig. 3, and also later in this work). This may explain the complete absence of coagulation in the samples where otherwise such clotting would have been expected.

So it may be concluded that also by adding the samples removed from the mixture undergoing coagulation to normal (unoxalated) plasma, it cannot be the presence of large amounts of *thrombin* which induces a clotting in so short a time as half a minute. The amounts of thrombin must be too small for that. It seems likely therefore that the prothrombin in the new plasma itself is undergoing activation after addition of the sample removed from the clotting mixture, and that the new-formed thrombin, in conjunction with the amounts added,

which of course also are still increasing in strength as the samples contain no oxalate, brings about the rapid coagulation. It is therefore not an *active* substance (thrombin) which induces the coagulation and is the cause of the autocatalytic reaction, but an *activating* substance, namely a substance which can activate prothrombin by converting it to thrombin, and which itself is present in plasma as an inactive precursor (prokinase) and may be transformed autocatalytically to the activating substance (thrombokinase) during the clotting. Further investigation will be necessary before this problem will be definitely solved, and according to orientating experiments this will be a difficult task due to the complexity of the phenomena and the lability of the substances in question[1]).

[1]) K. Laki, in a paper just received (Schw. med. Wschr. *74*, 13 (1944)), claims the isolation of a plasma component responsible for the autocatalytic reaction (a prothrombokinase).

Chapter III.

THE CLOTTING PROCESS

This chapter deals with the mechanism of the clotting process proper, *i.e.*, the conversion of fibrinogen to fibrin.

In the first section (A) the action of thrombin on plasma and fibrinogen is discussed. Section B deals with the influence of the ionic strength on the action of thrombin on fibrinogen. In section C the species specificity of fibrinogen is investigated taking the ionic strength into consideration. In the last section some questions regarding the significance of the ionic strength to enzymatic reactions in general are discussed.

A. The Action of Thrombin.

The relation between the amount of thrombin and the clotting time of the fibrinogen-containing mixture is of special interest with a view to the measurement of the activity of thrombin. Several papers have been published, in which this relation has been discussed, but so far no definite accordance has been arrived at (cf. the reviews by WÖHLISCH (*172*, *173*). Most authors have found more or less proportionality between the amount of thrombin and the reciprocal of the clotting time —*e.g.* MELLANBY (*110*, *112*), BLEIBTREU (*43*), QUICK (*133*), GLAZKO & FERGUSON (*85*) and HERBERT (*92*).

In our first investigations on thrombin (ASTRUP & DARLING (*21*, *22*)) we used oxalated ox plasma for the determination of thrombin activity, as we found a sufficiently accurate direct proportionality between the amount of thrombin and the reciprocal of the clotting time, when the clotting time did not exceed 2 minutes. The determinations were carried out as follows:

Of the thrombin solution 0.10 ml is placed in our usual clotting tubes (80 mm in height and 15 mm in diameter). Of oxalated ox plasma, which is kept at about 0° by standing in ice water, 1.0 ml is added to the thrombin solution by blowing out the pipette, so that an immediate mixing of the contents is obtained, but without blowing so violently that foaming occurs. The tube is placed immediately in a water-bath at 37° and the clotting time is determined. By making determinations on different dilutions of the thrombin solutions and plotting the amount of thrombin expressed in per cent of the original undiluted solution as abscissa and the reciprocal of the clotting time—expressed as reciprocal minutes: 60/t, where t is the time in seconds—as ordinate, a sufficiently straight line passing through the zero point was obtained.

Based on this, a *thrombin unit* (T.U.) was defined as the amount of active substance which, under the experimental conditions described, will clot 1.0 ml of oxalated ox plasma in 30 seconds.

Later, however, it was found that quite often it was impossible to obtain a straight line in this manner, and this question was therefore investigated more thoroughly.

The result of this investigation was that oxalated ox plasma was unsuited for the determination of thrombin activity, as the curves obtained varied considerably with the specimen of plasma employed. It was therefore decided to purify fibrinogen and try it as a substrate for the measurements, and these experiments have been published recently (ASTRUP & DARLING (*26*)). By removing prothrombin from ox plasma by adsorption with tricalcium phosphate and precipitation of the fibrinogen twice with ammonium sulphate, it was found that direct proportionality could be obtained between the amount of thrombin and the reciprocal of the clotting time.

However, the clotting of a purified fibrinogen solution is very sensitive to different influences—*e.g.* salt concentration, pH, protein concentration—and hence it was not possible to define a thrombin unit based on a fibrinogen solution.

It was necessary therefore to set up a standard for the thrombin, and such a sample was chosen; and the potency put at

12000 T.U. per gram of substance, which would give reasonable agreement with our earlier estimations. All determinations of thrombin potency therefore are now carried out by comparison with a solution prepared from this standard substance. The measurements have been described already (ASTRUP & DARLING (*26*)) and are carried out as follows:

50 mg of the thrombin standard are dissolved in 15 ml of physiological NaCl solution with the addition of one drop of octyl alcohol. After standing for an hour under occasional stirring, it is centrifuged or filtered, and the resulting clear solution contains 40 thrombin units per ml.

In the same manner, a suitable amount of a thrombin preparation, the strength of which is to be determined, is treated so as to give a solution with a strength comparable to the strength of the standard solution.

Serial dilutions of these two solutions are then made from 10 to 100 per cent of the original solutions by measuring suitable amounts (from 0.20 to 1.80 ml) of the solutions in small tubes and filling up to 2.00 ml with physiological NaCl solution.

The fibrinogen, as already mentioned, is prepared from oxalated ox plasma treated with tricalcium phosphate (Bordet plasma) by precipitating twice with ammonium sulphate and dialyzing in cellophane casings. This stock solution is diluted in a suitable manner with physiological sodium chloride solution and distilled water until 0.10 ml of the undiluted standard thrombin solution with addition of 1.0 ml of the fibrinogen solution shows a clotting time of about 10 seconds. During the measurements the fibrinogen solution is kept in ice-water.

The determination of the clotting times must be carried out with the utmost care, as only in this way is it possible to obtain a linear relationship. The clotting of the fibrinogen solution has not taken place when the first single threads of fibrin are formed, as this point is highly dependent on the quality of the fibrinogen solution, due to its varying content of impurities. The clotting time is taken as the point of time when fibrin threads commence uniting to form a real coagulum or to form larger threads or particles. The proceeding of the clotting varies somewhat from one fibrinogen solution to another, but as a

rule it is not difficult to observe when this conversion takes place. This time is the only well-defined juncture of the clotting, as the coagulation proceeds without any other sharp borderlines between its beginning and end. With a fibrinogen solution of very good quality the whole solution clots at this moment, but most often the complete clotting takes place a considerable time after the agglutination of the fibrin threads begins. Still, these differences between the properties of the fibrinogen solutions are of no importance to the determination of the strength of a thrombin solution.

The clotting is carried out in a water-bath at 37°, and for good visibility it is necessary to use a glass water-bath placed against a clear background (a window), as otherwise it is impossible to observe accurately the formation and agglutination of the fibrin threads. The tubes must be shaken regularly during the clotting, but violent and irregular shaking must be avoided if reproducible measurements are to be obtained.

In this manner the clotting time for all the thrombin dilutions is determined in seconds, taking the most potent solutions first. The dilution of the unknown thrombin preparation and its corresponding solution of the standard are measured immediately after each other, and not all the dilutions of one of the solutions before all the dilutions of the other, in order to avoid as far as possible the influence of an eventual alteration of the very unstable diluted fibrinogen solution.

From the clotting times t the value of $60/t$ is calculated and the reciprocal minutes thus obtained are used as ordinates for the plotting of a curve with the thrombin dilutions expressed in per cent of the concentrated solutions as abscissa. The clotting times usually lie between 10 and 60 seconds, and the average value of three determinations is used for the computation.

Fig. 6 shows two curves obtained in this manner. Of the unknown sample 75 mg was dissolved in 15 ml of physiological NaCl solution. By preliminary determinations with different solutions of a thrombin of unknown strength, an amount is determined that gives a potency of the resulting solution which lies in the neighbourhood of the strength of the standard solution, *i.e.* about 40 T.U. per ml. Such a solution is then used for the

final determination of the activity. It will be noticed that the points lie on straight lines; and by extrapolation it is found that 50 mg of the standard containing 12.0 T.U. per mg give a clotting time of 6.0 reciprocal minutes, while 75 mg of the new thrombin solution give the value 7.4. The activity of the sample is then calculated as

$$\frac{12 \cdot 7.4 \cdot 50}{6.0 \cdot 75} = 9.9 \text{ T.U. per mg.}$$

It is very fortunate that thrombin in a dry state seems to be a rather stable substance, so that it may be kept for a long time as a standard preparation. Should it deteriorate, methods are available for indirect checking of a new standard. Thus VOLKERT (*160*) (1943) has shown that one ml of citrated plasma (1:10) from normal rabbits on an average will inactivate 156 units of thrombin. As there seem to be only slight deviations from this mean value it is possible indirectly in this manner to define a thrombin unit.

Having now discussed the measurement of the action of thrombin and its standardization, some examples of the action on different substrates will be presented.

As already mentioned, most authors have found that the amount of thrombin is proportional with the reciprocal of the clotting time both of plasma and of fibrinogen solutions. Some authors, however, have found no such relation. Thus in Chapter

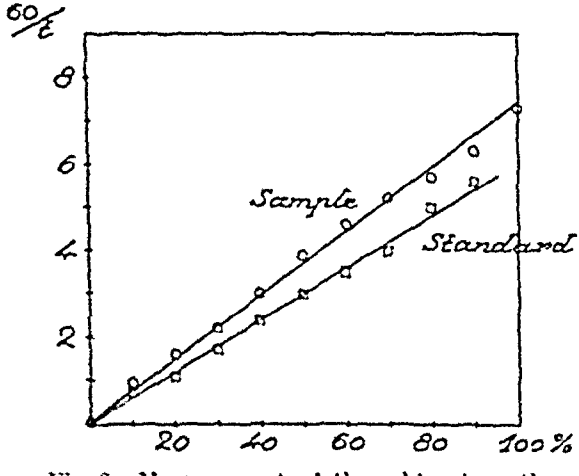

Fig. 6. Measurement of thrombin strength.

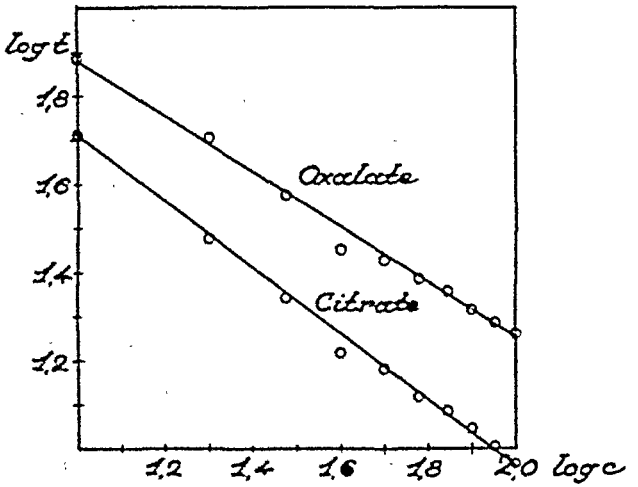

Fig. 7. Relation between thrombin content and clotting time of ox plasma.

II, section A, it was mentioned that the equation (1) used for the clotting by means of thrombokinase was used by BARRATT (*41*) and KUGELMASS (*105*) for expressing the action of thrombin on citrated and oxalated mammalian plasma.

As it was found that the reciprocity did not hold for plasma in our experiments, we have tried equation (1) and found it valid for this reaction. It is used in its logarithmic form (6).

$$\log c = \frac{1}{a}(-\log t - b) \qquad (6)$$

Two examples (oxalated and citrated plasma) are shown in Fig. 7. The value of a is respectively 0.62 and 0.75.

A considerable number of such measurements were carried out with plasma treated in various ways (dilution, buffer addition, adsorption, etc.). As a rule curves corresponding very well to equation (6) were obtained. The value of a was found between 0.5 and 0.75. In case of reciprocity a would have been equal to *one*.

Also fibrinogen solutions may give such results instead of reciprocity. This happens, for instance, with impure and deteriorated solutions, especially when made from plasma not treated beforehand with adsorption. Ammonium sulphate precipitation gives also in this respect the most suitable fibrinogen, but only after two precipitations. Fibrinogen precipitated by dilution

and acidification (MELLANBY fibrinogen) is unsuited for standardization (examples are shown by ASTRUP & DARLING (*26*)) and do not give reciprocity but curves corresponding to equation (6). In a paper just received double logarithmic curves corresponding to equation (6) were used by JAQUES (*99*) for the assay of thrombin.

B. The Significance of pH and Ionic Strength to the Action of Thrombin.

As is well known, the pH optimum for an enzymatic reaction is in many cases a very constant value, and is commonly used for characterizing the enzyme in question.

Thrombin has also been investigated in this respect by different authors (cf. WÖHLISCH (*172*, *173*)). Most of them have found a weak acid reaction to give the greatest velocity for the reaction between thrombin and plasma or fibrinogen. It must be pointed out that the optimum reaction of thrombin may be different from the optimum reaction for the transformation of prothrombin into thrombin by means of thrombokinase, and also from the clotting of blood plasma as a whole by thrombokinase, in which process the activation of prothrombin and the action of thrombin participate at the same time. Only the separate action of thrombin is investigated here.

TSUNOO (*156*) (1924), employing horse fibrinogen and phosphate buffer, finds the reaction optimum between pH 6.4 and 6.6, and with the same method EAGLE (*63*) (1937) finds pH 6.3-6.7. KUWASHIMA (*107*) (1923), using phosphate-NaOH buffer after CLARK, finds pH = 6.6

CRUT (*54*) (1935), however, obtains the best result at pH 7-8. He uses horse fibrinogen but does not state his buffer solutions definitely (Michaelis buffer, Clark and Lubs buffer scale). Also KUGELMASS (*104*, *105*) (1923) using addition of acid or alkali finds a pH optimum about 7.0.

HUDEMANN (*97*) (1940) uses ox fibrinogen and the following three buffers: phosphate buffer, diethylbarbiturate-acetate buffer after MICHAELIS (*117*) and glycocol buffer. She finds the

pH optimum in the region from 6.5 to 9.0, in which only small differences in clotting time are observed. Her curves, however, show that the results found for phosphate buffer are distinctly more acid than those found for the MICHAELIS buffer.

For $MgSO_4$-plasma with addition of acetic acid or ammonia DYCKERHOFF & KÜRTEN (*61*) (1936) find the optimum pH about 6.9. GLAZKO & GREENBERG (*87*) (1940), using ox fibrinogen, find optimum between pH 6.0 and 8.0.

In previous experiments (ASTRUP & DARLING (*25*) (1942)) the relation between pH and thrombin action was investigated on fibrinogens from ox, horse, chicken and man. The pH optimum here ranged from pH 6.1 to pH 6.7 and was different for the various fibrinogens. This reminds of the action of proteolytic enzymes, for which the optimum pH also to a certain degree is dependent on the nature of the substrate used. The experiments were carried out with fibrinogens precipitated after MELLANBY.

Later it was shown (ASTRUP & DARLING (*26*) (1942)) that MELLANBY fibrinogen was unsuitable for such measurements, and the whole subject was taken up for more thorough investigation. Here ammonium sulphate-precipitated fibrinogen and oxalated plasma were used; further, the salt concentration was varied. In these experiments new and unexpected reactions were disclosed, which will be presented below. Also the action on the different fibrinogens will again be dealt with. The results obtained have already been referred to in different reviews, thus in (*16*) and (*19*), and a short preliminary paper has been published (ASTRUP (*17*) (1942)).

In our first experiments we also found slightly acid reaction to be the best (*25*). But in trying to investigate the process in plasma, where the pH value was changed by the addition of acid, quite another result was obtained, as now the optimum value was found to be about pH 7.0-7.5. The following examples will illustrate these findings.

The determinations were carried out as described already. pH was measured, by employment of a quinhydrone electrode. An experiment with oxalated ox plasma and hydrochloric acid is described in Table III.

Table III.

Plasma ml	Hydrochloric NaCl ml	1-n HCl ml	pH	t (mean) sec.	60/t
20	1.0	0.0	7.76	14.6	4.1
20	0.9	0.1	7.39	11.9	5.0
20	0.8	0.2	7.06	11.7	5.1
20	0.7	0.3	6.75	12.0	5.0
20	0.6	0.4	6.43	12.8	4.7
20	0.5	0.5	6.25	16.8	3.6
20	0.4	0.6	5.97	29.8	2.0
20	0.3	0.7	5.73	67	0.9

The result is expressed graphically in Fig. 8. Other acids gave similar curves. Fig. 8 also shows a curve obtained with acetic acid in Bordet plasma from ox. Here the optimum is more sharp and lies between pH 6.7 and 7.0, while with oxalated plasma it was found to be between pH 6.7 and 7.5.

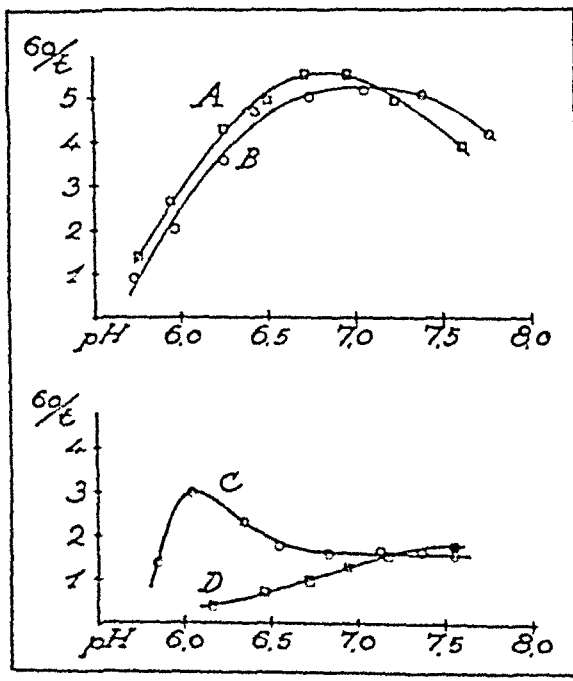

Fig. 8. Action of thrombin on ox plasma in relation to pH after addition of: Acetic acid (A); Hydrochloric acid (B); 0.2-m phosphate buffer (C); Hydrochloric acid and 0.6-m sodium chloride (D).

Earlier we found for ox fibrinogen (Mellanby) and phosphate buffer pH 6.4 (*25*). Therefore to 5 ml of the Bordet plasma was added 5 ml of 0.2–m phosphate buffer, and the mixture was examined (Table IV and Fig. 8).

Table IV.

pH of mixture	t (mean) sec.	60/t
7.56	38.3	1.6
7.37	36.7	1.6
7.13	36.6	1.6
6.83	38.1	1.6
6.55	33.5	1.8
6.35	26.1	2.3
6.05	20.1	3.0
5.84	44	1.4

Also for plasma, then, the optimum is found at a more acid reaction on employment of phosphate buffer than on addition of acid: in the cited case, pH 6.1 instead of pH about 7.0. From the results it is further evident that the activity of thrombin measured on plasma with addition of acid is far greater than on plasma with addition of buffer (with the exception of the most acid reaction). The difference in dilution in the two experiments is in this respect of no importance, as in earlier experiments we have seen that the plasma on dilution with physiological saline changes its reactivity against thrombin but very slowly; further, also other experiments with equal degree of dilution give the same result concerning the difference in pH optimum in the two cases.

So the experiments of earlier authors are only apparently conflicting, as the results will differ according to the experimental conditions during the measurements, and the most acid reaction will evidently be found by using phosphate buffer.

Now, it may be that the greater salt concentration in the experiments with phosphate buffer influences the formation of fibrin, as it is well known that neutral salts will retard the clotting. Still, by adding a constant amount of 0.6-m NaCl, which is found to give about the same clotting time at neutral

reaction as 0.2-m phosphate buffer, the optimum pH is not changed in a more acid region (see Fig. 8), but all the determinations give definitely longer clotting times (= lesser activity expressed as 60/t) than the normal plasma without addition. So the phosphate buffer curve appears to result from a simultaneous changing of the acidity and of the salt concentration.

As is well known, the physicochemical properties of proteins in solutions are greatly dependent on the *ionic strength* of the solution due to their numerous electrical charges as high molecular ampholytes. In the transformation of fibrinogen to fibrin by means of thrombin all the components are proteins. It is possible, therefore, that this reaction shows a dependency on the ionic strength of the solution far exceeding what would be expected for usual enzymatic reactions. So far nothing definite on the significance of ionic strength to enzymatic reactions has been published by any author, although in a few instances the ionic strength has been kept constant during the reaction. This possibility of the ionic strength influencing the reaction was therefore investigated.

The ionic strength μ of a solution is defined by equation (8)

$$\mu = \tfrac{1}{2} \Sigma m \cdot z^2 \tag{8}$$

in which m is the concentration of every single ion in question expressed in moles per liter, and z is its charge (or valency). In this manner not only the salt concentration as such is taken into consideration but also the charge of the ions in question.

In a phosphate buffer made, after SORENSEN, by mixing different amounts of equimolar concentrations of primary and secondary alkali phosphates, equal salt concentrations of the resulting buffer solutions are obtained, but the ionic strength varies to a great extent as the composition of the mixture and the pH value change. For practical purposes the ionic strength of the buffer solution may be calculated with sufficient accuracy by assuming the primary and secondary phosphate to be completely dissociated, and putting the ion concentrations equal to the concentrations of the monophosphate and the biphosphate ions. For the different mixtures the ionic strength may then

be calculated. For pure primary phosphate the ionic strength is found equal to the molarity, but for pure secondary phosphate it is three times the molarity. For any mixture the ionic strength therefore is found as a sum of its content of primary phosphate and 3 times its content of secondary phosphate. The result may be recorded either in a table or, preferably, as a curve, and such a curve is shown in recent publications, ASTRUP (*17*, *19*), where it is seen that as the pH changes, also the ionic strength changes considerably, and as a buffer is always used in a relatively large concentration in order to fulfil its function, the alterations in the ionic strength of the reaction mixture may also be considerable.

The investigation of the action of thrombin and fibrinogen was therefore continued with regard to the points set forth in the preceding, and it was found that the ionic strength is of utmost importance to this enzyme reaction, and that the difference between the curves obtained so far is due to alterations in the ionic strength. The investigations were continued along different lines: 1) First the significance of the ionic strength for the reaction was studied by using different salts. 2) Then the ionic strength was held constant while the pH was changed by diluting the buffer, and the curves were compared with the curves obtained with constant buffer concentration. 3) The pH was changed by addition of acid, and the ionic strength by addition of neutral salts. All the different experimental series gave the same result concerning the significance of the ionic strength to the reaction in question.

1. Employment of Different Salts.

It is well known that neutral salts inhibit more or less the action of thrombin on fibrinogen (see, for instance, the review by WÖHLISCH (*173*) and the paper by WEITNAUER, GRÜNING & WÖHLISCH (*168*)). Several authors have investigated the relation between the salt concentration and the clotting time (cf. GLAZKO & GREENBERG (*87*) and the references given there). Only on one occasion has the ionic strength been mentioned

in this connection, namely in the paper by GLAZKO & GREENBERG (57), who say:

"Upon using solutions of equivalent *ionic strength*, the same degree of inhibition was obtained with the oxalates, ferricyanides and ferrocyanides, but not with the salts of other anions. Therefore the inhibiting effect of anions is not solely a function of the ionic strength of the solutions. The effect of anions on the second phase of coagulation is negligible for univalent ions, and increases strongly with the valency. Similarly, the effect of cations on coagulation was found to be negligible for ions of low valence, but increased markedly for those of higher valence."

As will be shown, however, in contrast to GLAZKO & GREENBERG, we have found that the inhibitory action of neutral salts consisting of low-charged ions depends solely on the ionic strength, with the exception of ions of specific protein-denaturating nature, such as Ag^{+} and Cu^{++} ions. By increasing the charge (Al^{+++}, $FeCN^{----}$) these specific properties are increased, and the inhibition follows no longer the ionic strength. We thus found that while the low-charged ions as a rule act according to the ionic strength of the salt solution in question, with increasing charge the specific properties of the ion species become more and more pronounced and abolish the direct relation to the ionic strength.

First, several different salts were examined for ability to inhibit the thrombin action—in order to obtain information about their properties: either acting as neutral salts in general or possessing specific properties, making them unsuitable for our experiments.

To 5 ml ox *Bordet*-plasma were added 5 ml of different salt solutions, and the mixture was neutralized by addition of 1-n HCl or 2-n NaOH. The salt solutions here employed showed the calculated ionic strength $\mu = 0.6$, which—according to the orientating experiments already cited—showed a considerable inhibition of thrombin activity, namely to about $^1/_3$ or $^1/_4$. The small amounts of acid or alkali used for neutralization are assumed not to be of any importance to the total salt concentration. One ml of the mixture is added to 0.1 ml of the thrombin solution, and the clotting time is determined in the usual manner. The results are given in Table V:

Table V.

Salt solution	Molarity giving $\mu = 0.6$	Clotting time sec.	Remarks
Physiol. NaCl	—	9	Control
NaCl	0.6-m	36	
K J	0.6-m	>180	(see later)
Na_2HPO_4	0.2-m	27	
Na_3 citrate	0.1-m	26	
K_2 oxalate	0.2-m	33	
Li_2SO_4	0.2-m	34	
$Ca(NO_3)_2$	0.2-m	(—)	Precipitate
$BaCl_2$	0.2-m	(—)	Precipitate
$ZnSO_4$	0.15-m	(—)	Precipitate
$MgSO_4$	0.15-m	35	
$MnSO_4$	0.15-m	(—)	Precipitate
$AlCl_3$	0.1-m	(—)	Precipitate
$Al_2(SO_4)_3$	0.04-m	(—)	Precipitate
$KAl(SO_4)_2$	0.067-m	(—)	Precipitate
$La(NO_3)_3$	0.1-m	(—)	Precipitate
$Fe(NH_4)_2(SO_4)_2$	0.086-m	(—)	Precipitate
K_3 $Fe(CN)_6$	0.1-m	180	
$K_4Fe(CN)_6$	0.06-m	90	

From Table V it is seen that all the salts forming a precipitate with the plasma mixture before or after the addition of thrombin must be excluded; and this means that many interesting ions cannot be tried. Of the salts giving no precipitate, all the substances tried, with three exceptions, give about equal inhibition of the activity of thrombin, when solutions of equal ionic strength are used. This holds true for the following salts: NaCl, Na_2HPO_4, Na_3-citrate, K_2-oxalate, Li_2SO_4 and $MgSO_4$.

Two of the exceptions are potassium ferrocyanide and potassium ferricyanide. It is not unexpected, however, that in protein-containing solutions compounds with ions of this kind will react in a manner different from that of more simple neutral salts. It is interesting that highly charged anions, like ferrocyanide^{----} obviously act considerably less than highly charged cations, for instance Al^{+++}, but still more than the lesser charged ions. The clotting times after addition of these two salts are increased considerably in comparison with the normal

increase for solutions of ionic strength 0.6. The inhibitory properties of ferrocyanide have been studied especially by GLAZKO & GREENBERG (*87*) and GLAZKO & FERGUSON (*86*). (1941).

Another exception is potassium iodide, which in the amount employed completely inhibits the clotting, and thus shows properties entirely different from those of the other simple alkali salts investigated. As this was unexpected, other alkali halogenides were investigated, used as 0.6-m solutions ($\mu = 0.6$). This showed that the chlorides reacted as expected, and the bromides only showed minor deviations. The fluoride (NaF) accelerated the clotting, however, and iodide (NaJ,KJ) retarded it considerably. The action of sodium fluoride was so great that the solution containing equal parts of plasma and 0.6-m fluoride clotted in 7 seconds, while the mixture with physiological NaCl clotted in 9 seconds and with 0.6-m NaCl in 32 seconds. Sodium fluoride, however, may be looked upon as a complex substance and cannot therefore be compared with the other halogenides. It is also known as a precipitating medium for fibrinogen (cf. HUISKAMP (*98*) (1905)), and its effect may possibly be due to an acceleration of the precipitation of fibrin. When the mixture is left standing without addition of thrombin, a precipitation resembling a clotting takes place.

In constrast hereto, the iodides plainly inhibit the clotting. The solutions used (0.6-m) must be diluted about 5 times in order to obtain clotting results comparable with the times yielded by the other halogenides or simple neutral salts. This may be due to a denaturation occurring in the reaction, as KJ is shown by ANSON & MIRSKY (*2*) (1930) to be a denaturing agent.

The formation of fibrinogen by means of thrombin is thus an enzymatic process which, due to the delicate nature of the proteins taking part in the reaction, is subject to the most different disturbing influences, and the specific effect of several individual ions on this system is far more pronounced than the general properties of the salts expressed by their ionic strength. For investigation of the significance of the ionic strength, some

of the salts which show no anomalies are chosen and compared. As a rule sodium chloride and lithium sulphate are used.

Table VI shows the result of such a comparison. Ox Bordet plasma is used; to 10 ml of plasma different amounts of 0.6-m sodium chloride or 0.2-m lithium sulphate ($\mu = 0.6$) are added and diluted with distilled water to 20 ml. The ionic strength of the plasma itself is unknown, but may with reasonable accuracy be put as equal to a physiological NaCl solution (0.9 per cent = 0.15-m, $\mu = 0.15$). The ionic strength of the mixtures may therefore be calculated. The activity of the thrombin solution is as usual expressed as 60/t.

Table VI.

Salt solution ml	μ	NaCl		Li_2SO_4	
		t	60/t	t	60/t
0	0.075	6.1	9.8	5.1	11.8
2	0.135	7.1	8.5	7.0	8.6
4	0.195	12.4	4.8	10.2	5.9
6	0.255	24.0	2.5	19.8	3.0
8	0.315	35.3	1.7	39.1	1.5
10	0.375	48.6	1.2	68.0	0.9

As will be noticed, the potency of the thrombin solution is influenced to the same degree by different salt solutions of the same ionic strength. The results are expressed graphically in Fig. 9, in which the ionic strength is abscissa, and the thrombin activity expressed as reciprocal minutes is ordinate.

Fig. 10 shows similar experiments with a fibrinogen solution at about pH 6.8 and with employment of 0.6-m NaCl and 0.2-m Li_2SO_4. As with plasma, also here lithium sulphate seems to give a more straight line than sodium chloride.

In order to investigate the influence of salt concentration at different pH values, diluted HCl or NaOH is added to Bordet plasma whereafter salt solutions of varying ionic strength are added. Fig. 11 shows the result of such an experiment at pH respectively 6.0 and 7.7 and with employment of lithium sulphate. The action of the salt at the different pH values is of the same order, when due allowance is made for the difference

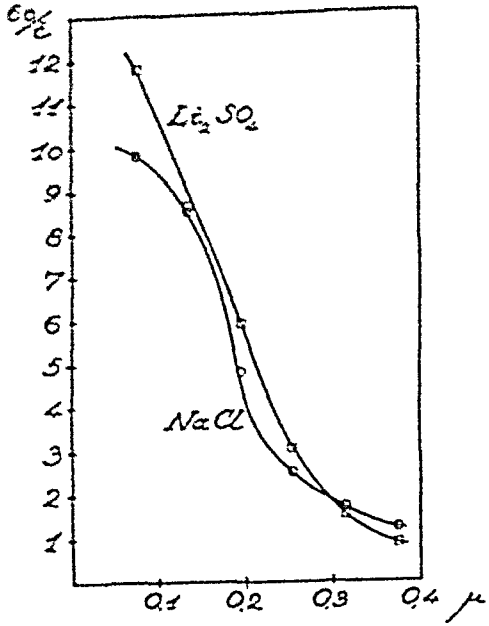

Fig. 9. Action of thrombin on ox plasma in relation to the ionic strength of the solution.

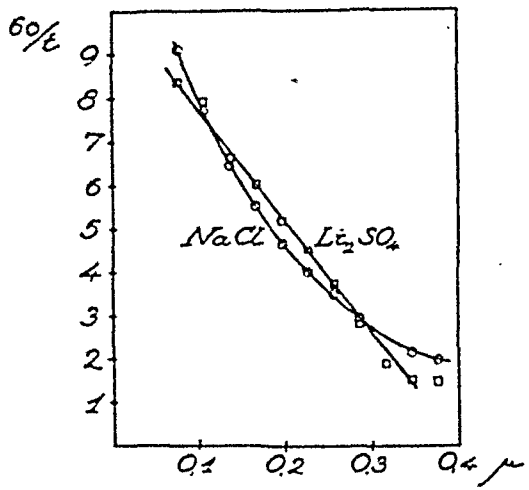

Fig. 10. Action of thrombin on ox fibrinogen in relation to the ionic strength of the solution.

of the clotting ability at the respective pH values. When the difference in pH values is not as great as in this experiment, the salt curves lie nearer each other. Sodium chloride yields similar results. Similar experiments were tried with fibrinogen,

but reproducible results could not be obtained, because in the fibrinogen solution with addition of dilute acid the character of fibrinogen in its reactivity with thrombin undergoes uncontrollable changes. This is due probably to a denaturing action of the acid on the purified fibrinogen which is very labile, unless it is stabilized by other proteins or salts. This property will be discussed later.

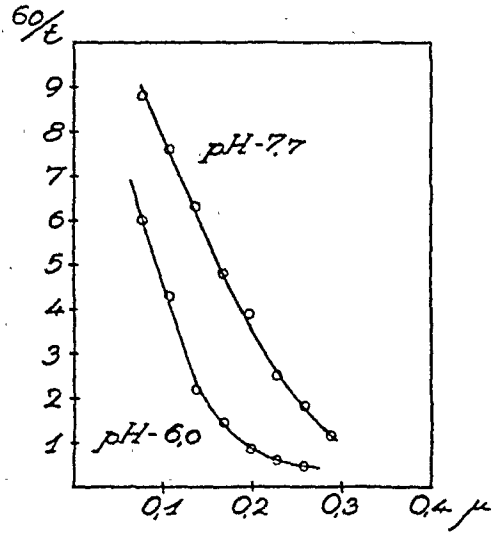

Fig. 11. Action of thrombin on ox plasma at different pH and varying ionic strength (Li_2SO_4).

All the experiments described here indicate that the clotting of fibrinogen by means of thrombin is an enzymatic process which is influenced very much by the concentration of simple neutral salts in the solution, expressed as the ionic strength, and that solutions showing equal ionic strength at the same pH also generally inhibit the clotting to the same extent.

2. *Constant Ionic Strength and Varying pH.*

After completion of the experiments concerning the effect of neutral salts on the clotting of fibrinogen, the studies on the pH optimum were taken up again.

First the curves obtained by diluting plasma or fibrinogen with solutions of phosphate buffer of respectively constant molar concentration and constant ionic strength were compared.

The solutions of constant ionic strength were made by diluting the phosphate buffer with water in the proportion calculated from the curve for the variation of the ionic strength with the pH. In this manner the curve published recently was obtained (*17, 19*). Another example is shown in Fig. 12.

From these curves which were obtained on plasma it is seen that by adding phosphate buffer of constant molar concen-

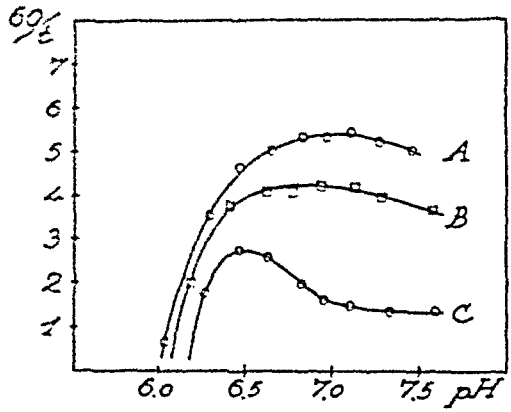

Fig. 12. Action of thrombin on ox plasma in the presence of phosphate buffer. *A:* Equal volumes of Bordet plasma, physiological NaCl solution and phosphate buffer ($\mu = 0.2$). *B:* One volume of plasma, two volumes of phosphate buffer ($\mu = 0.2$). *C:* Equal volumes of plasma, physiological NaCl and 0.2-m phosphate buffer.

tration an optimum pH is found at about pH 6.5, while by using phosphate buffer of constant ionic strength the optimum is found about pH 7.0, in accordance with the results obtained by changing the pH by addition of diluted acid. The position of the optimum pH is therefore dependent on the ionic strength of the solution, and may be changed at will by changing the ionic strength.

On changing the pH value by addition of diluted hydrochloric acid to plasma the pH optimum is not changed, but only flattened, by increasing salt concentration of the solutions. This is shown by the three curves of Fig. 13, with employment of Bordet plasma and solutions of lithium sulphate.

In carrying out the same series of experiments on solutions of fibrinogen some difficulties are met with. Reproducible results are obtained only with the buffer experiment, while

the experiments with addition of acid must be given up, because of the instability of the fibrinogen solutions under these circumstances. Fig. 14 shows two curves obtained by adding phosphate buffer of respectively constant molarity and constant ionic strength to a fibrinogen solution. The results are in accordance with the results obtained with plasma concerning the position of the respective pH optima.

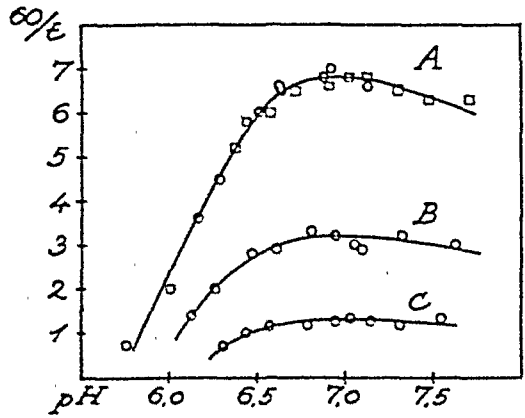

Fig. 13. Action of thrombin on ox plasma by addition of hydrochloric acid and in the presence of equal amounts of Li_2SO_4 solutions of different concentrations. *A:* 0.05-m. *B:* 0.10-m. *C:* 0.15-m.

The instability of unbuffered fibrinogen solutions is the cause of the poor results obtained by the addition of acid. This is shown by the following experiment: A fresh fibrinogen solution, prepared as usual for the determination, is placed in ice-water and the pH is followed for two hours. The initial pH is 8.47; after standing for 15 minutes, it is 7.82, and after 50 minutes 7.23. After two hours it is 6.35. So even at 0° the fibrinogen changes continually, and the solution becomes more and more acid. It is therefore no wonder, that unbuffered solutions give inconstant results, and that it is necessary to add a buffer solution in order so obtain a constant pH value in the solution. Also in these solutions, of course, the fibrinogen changes its properties, but this does not interfere with the hydrogen ion concentration of the solution, and hence it is of no interest in this respect.

This instability of unbuffered solutions is also of importance

to the determination of the strength of thrombin solutions, and shows that it is best to use buffered solutions for this purpose. For this phosphate buffers may be used as done in the experiments reported here, but it is better to use the diethyl barbiturate buffer of MICHAELIS (*116*). Under certain circumstances the determination of the clotting point is difficult in phosphate buffers, while the barbiturate buffer does not seem

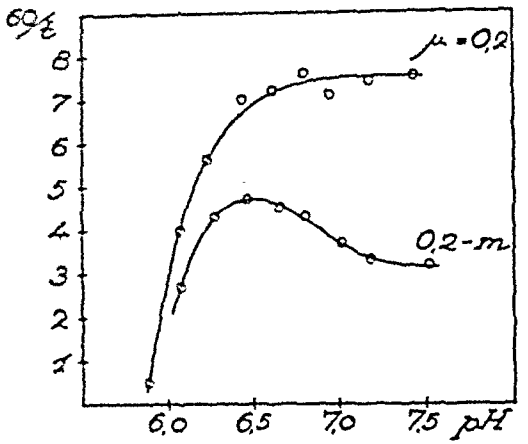

Fig. 14. Action of thrombin on ox fibrinogen by addition of phosphate buffer.

to interfere with the determinations. Especially, this holds true for the determination of the thrombin activity, which is to be carried out during the measurements of antithrombin (see later).

3. *Varying pH and Ionic Strength.*

The results obtained in the two preceding sections may be verified in another manner, namely by changing in the same plasma system the pH by addition of acid and the ionic strength by addition of neutral salts. Such experiments were therefore carried out on plasma, while, owing to the difficulties just mentioned, purified fibrinogen solutions were not tried. Fig. 15 shows the results of two such experiments, one with employment of different concentrations of sodium chloride, the other with lithium sulphate. To 10 ml plasma are added 20 ml of a mixture of 0.6-m NaCl or 0.2-m Li_2SO_4 and distilled water, and then 1-n HCl is added until the desired pH is reached. In Table VII

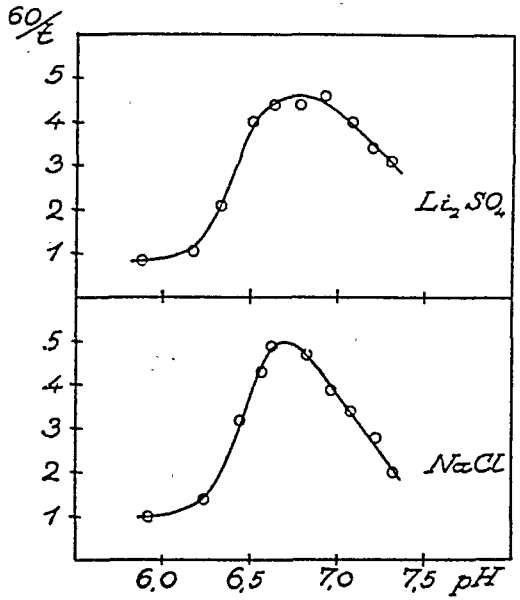

Fig. 15. Action of thrombin on ox plasma by addition of hydrochloric acid and varying amounts of 0.6-m NaCl or 0.2-m Li_2SO_4 (see Table VII).

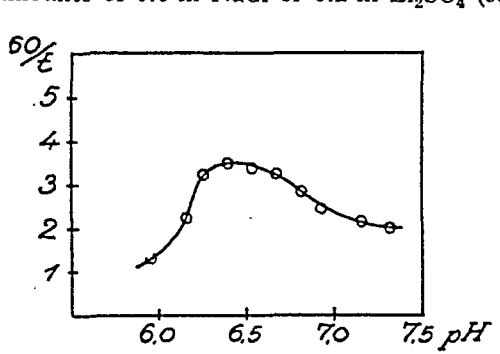

Fig. 16. Action of thrombin on ox plasma by addition of hydrochloric acid and differently varying amounts of 0.6-m NaCl (see Table VIII).

an example of such an experiment is recorded. In this experiment from 4.5 to 9.0 ml 0.6-m NaCl are used in increasing amounts with increasing pH value. The curves obtained show a shifting of the pH optimum to the acid side, just as is the case on employment of phosphate buffer of constant concentration. By using sodium chloride in irregularly increasing amounts it is possible to obtain curves which still more resemble the curves obtained with common phosphate buffer. An example of this is recorded in Table VIII and in Fig. 16. It shows quite the same features as obtained with phosphate buffer.

The dependence of the fibrin formation on the ionic strength has thus been demonstrated in various ways, which all give concordant results.

Table VII.

1-n HCl ml	0.5-m H_2SO_4 ml	H_2O ml	t mean	60/t	pH
0.00	9.0	11.0	19.5	3.1	7.30
0.03	8.5	11.5	17.8	3.4	7.20
0.06	8.0	12.0	15.2	4.0	7.08
0.09	7.5	12.5	13.0	4.6	6.92
0.12	7.0	13.0	13.5	4.4	6.78
0.15	6.5	13.5	13.6	4.4	6.63
0.18	6.0	14.0	15.0	4.0	6.51
0.21	5.5	14.5	28.5	2.1	6.33
0.24	5.0	15.0	57.4	1.04	6.17
0.27	4.5	15.5	69.4	0.86	5.88

Table VIII.

1-n HCl ml	0.6-m NaCl	H_2O ml	t mean	60/t	pH
0.00	9.0	11.0	29.8	2.00	7.32
0.03	8.75	11.25	28.1	2.13	7.16
0.06	8.50	11.50	24.4	2.46	6.93
0.09	8.25	11.75	21.0	2.86	6.81
0.12	8.00	12.00	18.5	3.24	6.67
0.15	7.75	12.25	17.6	3.40	6.54
0.18	7.25	12.75	17.2	3.50	6.40
0.21	6.75	13.25	18.6	3.23	6.26
0.24	6.00	14.00	27.0	2.22	6.16
0.27	5.00	15.00	46.1	1.30	5.96

C. Thrombin Action, Ionic Strength and Species Specificity of Fibrinogen.

As already pointed out, our investigation concerning the significance of the ionic strength to the clotting of fibrinogen had its origin in the studies on the pH optimum of the process and the species specificity of fibrinogens published recently (*25*). After completion of the experiments on the effect of the ionic strength on the fibrin formation, these studies were again taken

up in order to elucidate the reaction in the light of the new knowledge concerning the influence of neutral salts upon the process.

These experiments are carried out with employment of fibrinogen prepared by ammonium sulphate precipitation according to (*26*) instead of acid-precipitated MELLANBY fibrinogen. Oxalated horse plasma and citrated human plasma are used, and in most of these experiments Bordet plasma is made as

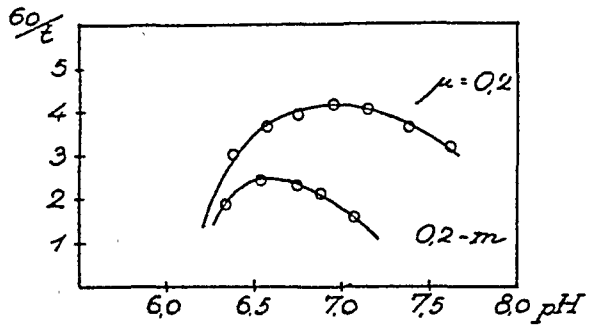

Fig. 17. Action of ox thrombin on horse fibrinogen by addition of phosphate buffer.

described previously (*26*). From chicken plasma Bordet plasma is made as described in (*8*). For precipitation of fibrinogen from human plasma it is necessary to increase the concentration of saturated ammonium sulphate to 0.33. This in itself is a sign of species specificity (cf. (*25*, *26*)). In some experiments chicken plasma and oxalated human plasma were used for fibrinogen preparation without preceding treatment with tricalcium phosphate. The experiments were carried out as described in the preceding, and some results will be presented here in the form of curves. On every fibrinogen solution two experiments were made, one with phosphate buffer of constant molarity, the other with phosphate buffer of constant ionic strength.

Fig. 17 shows an example with horse fibrinogen. The influence of the salt concentration is clearly seen, and the pH optimum for constant ionic strength ($\mu = 0.2$) of the phosphate buffer added lies about neutral reaction (pH = 7.0), while with addition of 0.2-m phosphate buffer the pH is found to be 6.6. In five experiments of this kind the pH for constant ionic strength

varied from 7.0 to 7.1, while with 0.2-m buffer it ranged from pH 6.6-6.8. The former investigations (*26*) gave pH 6.4 for constant molarity; but, as the pH optimum under these conditions may be changed with the concentration of the phosphate buffer in relation to the total salt concentration, this value may vary considerably with the experimental conditions

For human fibrinogen an example is shown in Fig. 18, from

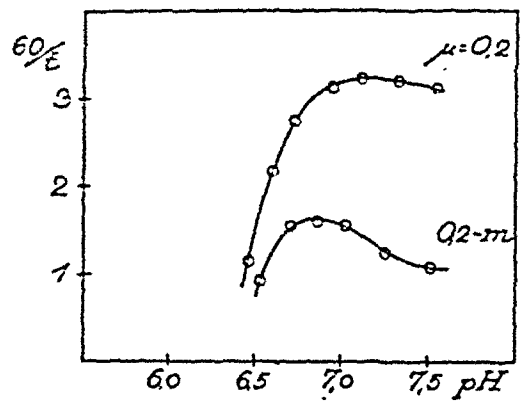

Fig. 18. Action of ox thrombin on human fibrinogen by addition of phosphate buffer.

which the pH optimum for constant ionic strength is found to be about 7.2, and for constant molarity pH = 6.8. In four experiments of this kind the pH for constant ionic strength varied from pH 7.2 to 7.6, and with constant molarity from pH 6.8 to 7.0. The previous experiments under these conditions gave pH = 6.7.

The corresponding experiments with ox fibrinogen have been described already under the experiments carried out for elucidation of the influence of the ionic strength on the clotting process; they need not be repeated here. The optimal pH for constant ionic strength ranged from 7.0 to 7.5, while for constant molarity it varied between pH 6.4 and 6.7. Earlier experiments had given pH 6.4.

Chicken fibrinogen showed immediately its difference from the other fibrinogens, as it was impossible with chicken fibrin-

ogen to obtain as small clotting times as used in the experiments with mammalian fibrinogen. While for ox fibrinogen the clotting times were as low as 8–10 seconds, a chicken fibrinogen solution made in the same manner as a rule clotted in about 25 seconds. With chicken fibrinogen, however, it was easier to obtain good determinations of the longer clotting times than with ox fibrinogen. An example of experiments on chicken

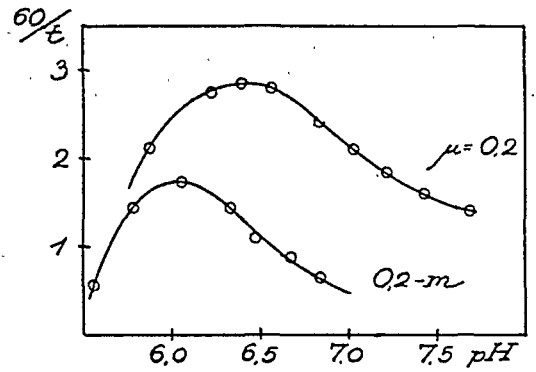

Fig. 19. Action of ox thrombin on chicken fibrinogen by addition of phosphate buffer.

fibrinogen is shown in Fig. 19. In both cases the pH optima are found in a considerably more acid region than usual, namely for constant ionic strength at pH 6.4 and for constant molarity at pH 6.0. In five such experiments the optima varied respectively between pH 6.4-6.5 and pH 6.0-6.2. Earlier experiments had shown pH 6.1. It is interesting that while the real pH optimum, as it is found by making the ionic strength constant in different ways, for the mammalian fibrinogens investigated is always found near the neutral point, and thus lies in the neighbourhood of the natural pH of the blood, this does not seem to apply to the formation of chicken fibrin, as the real pH optimum here is found about pH 6.4.

The results for the pH optima for the different fibrinogens at constant ionic strength and constant molarity may be summarized in the approximate values shown in Table IX.

So the experiments here described verify not only the species specificity of fibrinogen as disclosed in its reactivity with

Table IX.

Fibrinogen	pH at constant ionic strength	pH at constant molarity
Man	7.5	6.9
Horse	7.0	6.7
Ox	7.0	6.5
Chicken	6.4	6.1

thrombin at different pH values; they also confirm the earlier results concerning the significance of the ionic strength to the clotting of fibrinogen.

D. Considerations Concerning the Influence of Ionic Strength upon Enzymatic Reactions.

It is not the place here for a general treatment of the theoretical aspects of the influence of ionic strength on enzymatic reactions. The process here investigated, however, seems to be the first enzymatic reaction for which the significance of the *ionic strength* has been definitely demonstrated. The opportunity may therefore be taken to consider the possible significance of the ionic strength to such processes.

It has been common practice for some years to use solutions of constant ionic strength for the investigation of physicochemical properties of proteins, in order as far as possible to eliminate the influence of variations in the interionic forces on the properties studied. In enzyme chemistry, however, generally no regard has been paid to this question, and the influence of ionic strength on enzymatic reactions has not yet been investigated in detail. Only in a few cases, mostly in American publications, buffers of constant and stated ionic strength have been used for studies on enzymes, but in no instance has the dependence of the reaction in question on the ionic strength of the solution been investigated separately. Only LUNDSTEEN (*109*) (1936) finds that the clotting of caseinogen by rennet is influenced by the ionic strength of the solution, and that presumably it is the precipitation of casein and not its formation which is altered. However, he makes no further comment on this phenomenon.

The term "ionic strength" defined according to equation (8) was introduced by G. N. LEWIS and is commonly used throughout the English-speaking countries. In German often the term "ionale Konzentration" (N. BJERRUM) is used. It is denoted with the sign Γ and defined by equation (9):

$$\Gamma = \Sigma m \cdot z^2 = 2\mu \qquad (9).$$

The variation in the ionic strength of 0.1-m phosphate buffer in relation to pH has already been mentioned, and a curve illustrating this has been published recently (ASTRUP (*17*, *19*)). In the same paper other curves show the relation between pH and ionic strength of 0.1-m acetate buffer, 0.1-m citrate buffer and citrate buffer according to SORENSEN. In the last case regard is paid to a varying sodium chloride content. The curve shows that the ionic strength for SORENSEN citrate buffer is constant between pH 5 and 9, but unfortunately the buffer capacity in the physiologically most interesting region (pH 6.5-9) is only small, so that the buffer cannot be used in this region. All the curves are calculated with the assumption of completely dissociated salts, while no regard is paid to the amount of free acid (acetic acid or citric acid), as the hydrogen ion concentration is of no importance for the ionic strength in proportion to the concentration of salts in the pH region used for enzyme studies. In such investigations the salt concentration and the buffer capacity must always be relatively large in order to give sufficient buffer action; and further the accuracy of biochemical measurements is relatively restricted, at any rate in comparison with the accuracy with which physicochemical measurements may be carried out.

For acetate buffer the ionic strength of the buffer usually employed ranges from about zero to the molarity of the solution. For citrate buffer according to SORENSEN μ ranges from 0.067 to 0.3, while for 0.1-m phosphate buffer, as already mentioned, it varies between 0.1 and 0.3.

Thus it is seen that all the buffers most commonly used show a very great variation of the ionic strength in relation to pH when they are made according to the usual methods.

In the preceding it was shown that the clotting of fibrinogen

by means of thrombin is not only dependent on the pH value of the solution but also on the ionic strength of the mixture. The lacking understanding of this relationship had been the cause of conflicting results. In general the ionic strength is not taken into consideration in enzymatic investigations, although in a few cases buffers of constant ionic strength have been used. From the results cited it is seen, however, that due to the great variations in the ionic strength of the buffer solutions commonly used for biochemical studies and to the unsettled significance of ionic strength to a given enzymatic reaction it is necessary to take this possibility into consideration. Especially in cases where different pH optima are found under various experimental conditions it is essential to pay attention to this matter. Only curves obtained at constant ionic strength may be assumed to give the real pH optima. This may be obtained in various ways—in most cases with sufficient accuracy by using diluted acid or alkali for changing the pH of the solutions.

It is very unfortunate that the buffers generally used for biochemical investigations show such great variations in their ionic strength, and thus the methods for obtaining constant ionic strength in buffer systems become of importance.

The method most commonly used is to dilute the buffer mixture in question with distilled water, in such a proportion that the ionic strength is held constant, when the composition of the mixture and hence the pH value is changed. From the curves shown in the papers already mentioned (*17*, *19*) it is easy to calculate the amount of water which is to be added. This method is of particular value for phosphate buffers. Here it is easy from a mixture of 0.1-m solutions of primary and secondary phosphate to obtain buffer solutions with ionic strength $\mu = 0.1$ by suitable dilution.

Instead of diluting with water, and thus be restricted to buffer solutions of relatively low ionic strength, it is also possible to *add* solutions of neutral salts to the mixtures and in this manner increase the ionic strength in solutions with too low values. This method may also be used for phosphate buffers, but is of still more value in buffers containing uni-univalent electrolytes, for instance acetate buffers. In such buffers the ionic strength may vary from about zero to the molarity of

the solutions, and by addition of neutral salts it is possible to increase the ionic strength to a constant value. In fact it is easy in buffers of this type by simple means to obtain constant ionic strength, and in some buffer compositions this principle has been used. It will be discussed below in connection with the discussion of the general properties of uni-univalent buffers.

Another method of obtaining constant ionic strength is to use a constant and relatively large amount of neutral salt and add varying and small amounts of the buffer. In this manner the contribution of the buffer salts to the ionic strength of the solution is small in comparison with the significance of the neutral salts. For biochemical studies, however, this method is not very satisfactory. As a rule it is necessary in such experiments not to use too small amounts of buffer, and the amount of neutral salt added must therefore be relatively large. Many enzyme reactions and reactions in which proteins take part are largely influenced by the concentration of neutral salts—for which effect in enzyme chemistry the term "salt effect" is used—and a large salt concentration means that the process is carried out under unphysiological conditions. Therefore it is not very suitable in such investigations to use this method to obtain a constant ionic strength.

When constant ionic strength is desired the most suitable buffers are solutions containing uni-univalent electrolytes. In mixtures of such electrolytes it is possible by various simple means to obtain constant ionic strength.

A buffer of this type, much used in biochemical investigations, is the diethylbarbiturate buffer described by MICHAELIS (*116*). The buffer is prepared by mixing varying amounts of 0.1-m sodium diethylbarbiturate with varying amounts of 0.1-m hydrochloric acid. The resulting mixture may be looked upon as containing undissociated diethylbarbituric acid, completely dissociated sodium diethylbarbiturate and completely dissociated sodium chloride corresponding to the amount of hydrochloric acid added. The amount of diethylbarbituric acid also corresponds to the amount of hydrochloric acid added, and as the barbituric acid is of no importance to the ionic strength, in this respect the only alterations which have taken place in

the solutions are that some sodium diethylbarbiturate has been replaced by the corresponding amount of sodium chloride. As these two salts with sufficient accuracy give the same ionic strength, it is seen that the ionic strength may be calculated from the original sodium diethylbarbiturate content of the solution without paying regard to the amount of hydrochloric acid added. The results are calculated in Table X.

Table X.

0.1-m Barbiturate ml	0.1-m HCl ml	pH	Ionic Strength μ
5.10	4.90	(6.40)	0.0510
5.14	4.86	(6.60)	0.0514
5.22	4.78	6.80	0.0522
5.36	4.64	7.00	0.0536
5.54	4.46	7.20	0.0554
5.81	4.19	7.40	0.0581
6.15	3.85	7.60	0.0615
6.62	3.38	7.80	0.0662
7.16	2.84	8.00	0.0716
7.69	2.31	8.20	0.0769
8.23	1.77	8.40	0.0823
8.71	1.29	8.60	0.0871
9.08	0.92	8.80	0.0908
9.36	0.64	9.00	0.0936
9.52	0.48	9.20	0.0952
9.74	0.26	9.40	0.0974
9.85	0.15	9.60	0.0985
9.93	0.07	(9.80)	0.0993

The pH values in parenthesis are not fully reproducible, according to MICHAELIS. The ionic strength in relation to pH is shown graphically in Fig. 20. It varies between 0.05 and 0.1.

In the same way the acetate buffer, prepared as usual, may be treated, and in this manner the curve published recently was obtained.

It is easy to see that in buffers of this type, containing salts

of monovalent weak acids, it is possible in a very simple manner to devise buffer mixtures with constant ionic strength by using a constant amount of the salt in question (sodium diethylbarbiturate, sodium acetate) adding varying amounts of hydrochloric acid and diluting to equal volume. The amount of the sodium salt present added to the amount of sodium chloride

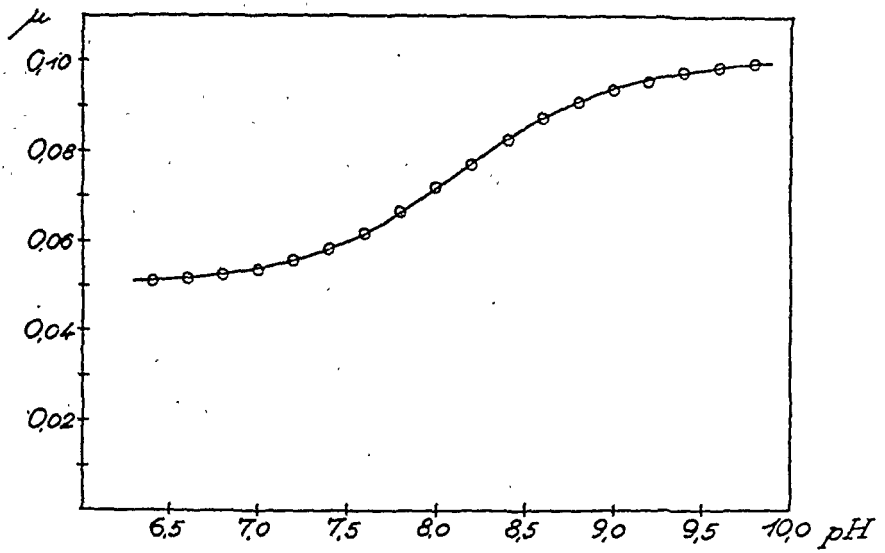

Fig. 20. Ionic strength of diethyl barbiturate buffer.

formed, expressed as moles per liter, will always be the same, and thus the ionic strength will be constant too. Some of the sodium salt of the acid will be replaced by the corresponding amount of sodium chloride, and in most cases this probably will be of only minor importance, at any rate in comparison with a corresponding variation in the ionic strength. However, it is possible that in certain cases specific properties of different ions play a rôle not to be disregarded, and in such cases it is therefore necessary to have this possibility in mind.

In a paper on the precipitation of caseinogen by rennet, MICHAELIS & MENDELSSOHN (*118*) (1913) used an acetate buffer containing a constant amount of sodium acetate and varying amounts of acetic acid (see also MICHAELIS & RONA (*119*) (1910)). However, it is better to change the pH value by addition of hydrochloric acid as rather large amounts of acetic acid must be used when the pH has to be changed to a more acid region,

and the large amount of acetic acid present then may change the properties of the solutions employed.

Later MICHAELIS (*117*) has described a modified acetate-diethylbarbiturate buffer, with which it is possible to cover a large pH region and to have constant ionic strength. According to the principle just mentioned, to a solution equimolar in sodium acetate and sodium diethylbarbiturate varying amounts of hydrochloric acid are added. This buffer seems to be convenient for many purposes.

Instead of using alkali salts of weak acids as buffers, hydrochlorides of weak bases may be used; especially the imidazole derivatives (glyoxalines) introduced by KIRBY & NEUBERGER (*103*) (1938) seem promising for biochemical studies. Unfortunately they are not easily prepared. These salts are uni-univalent electrolytes and it is easy to obtain solutions of constant ionic strength by using constant amounts of the hydrochloride of the base, adding varying amounts of sodium hydroxyde and diluting to equal volume, corresponding to the principle adopted for weak univalent acids. KIRBY & NEUBERGER have made an extensive investigation on the properties of these substances as buffers and from their tables it is possible to calculate the pH of any mixture and at varying ionic strength of the solutions. Imidazole was used by MERTZ & OWEN (*114*) for studies on blood clotting, as it does not react with the calcium ions, but unfortunately they mixed equal amounts of the base with varying amounts of hydrochloric acid, so that diluting to equal volume did not give solutions of equal ionic strength.

On phosphate and acetate buffers extensive investigations have been made by COHN and co-workers (COHN (*52*), COHN, HEYROTH & MENKIN (*53*), GREEN (*88*)), and BJERRUM & UNMACK (*42a*) have investigated buffers based on phosphoric acid, citric acid and glycine. From their tables and curves it is possible to calculate the composition of a buffer for any pH value and ionic strength of the solution. These salts, together with the imidazoles, are the only buffers used in biochemistry on which extensive investigations have been carried out.

In order to obtain some information of the significance of the ionic strength for the properties of buffers a model experi-

ment was performed recently by BRODERSEN (*48*). He measured the potential of a ferro-ferricyanide electrode in buffers of different composition. The potential of such an electrode with a not too acid reaction is independent of the pH of the solution, and therefore the salt effect may be investigated separately. He found that the potential was greatly influenced by the ionic strength of the solution, but also buffers diluted to constant ionic strength showed deviation, almost as large as buffers with varying ionic strength. This deviation was shown to be due to the various properties of the different ion species in the mixture, and a constant potential was obtained only by using constant and relatively large amounts of a neutral salt and only a small concentration of the buffer, *i.e.* under circumstances generally not existing in most biochemical investigations. So it should always be kept in mind in such investigations that, whenever possible, the buffer concentration must be kept low and a constant and relatively high concentration of neutral salt must be present in the solutions. Only under such conditions deviations due to varying ionic strength and different ionic composition may be eliminated. In the light of his findings BRODERSEN discusses the properties of buffers to be used in biochemistry.

It is important that even in such a simple reaction as the oxidation of ferrocyanide to ferricyanide studied by BRODERSEN:

$$Fe\ (CN)_6^{----} \rightleftarrows Fe\ (CN)_6^{---} + e^-$$

it is not possible to eliminate differences in the interionic forces influencing the activities of the ions in question by using solutions of equal ionic strength when the composition of the buffer mixtures is changed. The individuality of the different ions in the solution influences the reaction to a very significant degree and in a manner not taken into consideration in the calculation of the ionic strength, which term only depends on the valency and concentration of the ions in question.

Now, as the reaction mentioned takes place between tetravalent and trivalent ions, we may expect that the reaction to a large extent depends on the ionic strength of the solution, in the expression of which the valency is raised to the second

power. Also the interionic forces due to the individuality of the different ions and not taken into consideration in the ionic strength must play a similar increased rôle in reactions concerning ions of higher valency. Furthermore, it is well known that the activity of the ions in more concentrated solutions, such as is generally used in biochemistry, does not simply depend on the ionic strength, and that a corrected equation has to be used for such solutions (see, for instance, the investigations already mentioned by COHN and co-workers). In a not very dilute solution the activity of an ion with high valency may be only a fraction of the activity of a monovalent ion in the same solution. This is expressed through the two well-known equations (10) and (11), of which the first is valid for low concentrations only ($\mu \leqq 0.01$) and the second for more concentrated solutions ($\mu \leqq 0.1$). As usual, z is the valency of the ion in question and f its activity coefficient in watery solution at 20°.

$$-\log f = 0.5 \cdot z \cdot \sqrt{\mu} \tag{10}$$

$$-\log f = 0.5 \cdot z \cdot \frac{\sqrt{\mu}}{1+\sqrt{\mu}} \tag{11}$$

In biochemical investigations in general proteins take part, either alone as an enzyme or both as enzyme and substrate, or finally as enzyme, substrate and reaction product. As the protein contains a large number of both positive and negative electrical charges it is no wonder that reactions in which proteins take part are greatly influenced by the interionic forces, and especially that the ionic strength of the solution must be taken into consideration. From what has been said already it is also obvious that the behaviour of such systems may not be described in terms of the ionic strength alone.

As a rule the ionic strength is taken into consideration in physico-chemical investigations of proteins, and in many cases it is possible in this manner to obtain satisfactory results in solutions of different composition, for instance in buffers of different nature. But often the individuality of the ion species also plays a rôle and modifies the results obtained to a certain degree. This, for instance, is the case in determination of iso-

electric points, mobility in an electric field and so on. In not too concentrated salt solutions, however, the mere maintenance of a constant ionic strength is usually sufficient to obtain results which may be compared with each other with reasonable accuracy.

As already mentioned, enzyme reactions have not hitherto been investigated in detail with respect to these questions. This circumstance is due probably to the fact that the accuracy of the measurement of reaction velocity in enzymatic processes is relatively small in comparison with the measurement of the different physico-chemical properties of proteins just mentioned. Further it is possible that only certain enzyme reactions will show so great a dependence on the interionic forces (expressed as the ionic strength) that it will exceed sufficiently the accuracy with which the measurement is carried out. It is possible that only a few reactions may show a large influence of the ionic strength, and that the large majority of enzyme reactions is only to a slight extent dependent on the ionic strength. The reactions for which the greatest significance of the ionic strength is to be expected are the reactions in which a protein is transformed into another protein under the influence of an enzyme, and such a reaction is, for instance, the formation of fibrin from fibrinogen by means of thrombin. So, it is no wonder that just this process should show such relations as described in the preceding sections of this chapter.

In this case three proteins take part in the reaction, the substrate fibrinogen, the enzyme thrombin and the reaction product fibrin. Further, after passing through a soluble form "profibrin", the fibrin is precipitated as a gel during the reaction; in fact, it is possible that it is the last of these reactions, the transformation of the soluble profibrin into the insoluble fibrin, which is the part of the process most influenced by the ionic strength. This probably makes the action of thrombin to an enzyme reaction, which in a considerably higher degree than other reactions, even such which also take place between three proteins, depends on the ionic strength of the solution.

In a preceding section it was shown that the action of thrombin shows different pH optima according to the origin of the

fibrinogen used as substrate. For many enzymes the pH optimum is a rather fixed point, but it is well known that for proteolytic enzymes the pH optimum in some degree depends on the protein used as substrate and varies with the isoelectric point of it. As mentioned already, the reaction optima for the thrombin action vary between pH 6.4 and pH 7.5, depending on the fibrinogen species employed, when the investigations are carried out in solutions of constant ionic strength, and for the fibrinogens used in our investigations. The isoelectric points of the different fibrinogens are not known. We have made some experiments in this direction, but as fibrinogen is a very labile protein, great technical difficulties are met with in the preparation of sufficiently pure solutions for such measurements, and so far we have not succeeded in these investigations.

For thrombin itself the isoelectric point is found about pH 4.4 (SEEGERS (*137*), ASTRUP & DARLING (*22*)) and is thus far removed to the acid side from its reaction optimum, at which point thrombin therefore will carry large, negative electrical charges. So, probably thrombin exerts enzymatic activity only as an anion. As thrombin is thus highly electrically charged at the reaction optimum, it is understandable that the dependence on the ionic strength is so great, and it is reasonable to assume that other enzymatic reactions, where the isoelectric point of the enzyme lies far away from the reaction optimum of the enzymatic process, also may show such relations. Of course, the same must be the case when it is the isoelectric point of the substrate and not that of the enzyme which is far removed from the reaction optimum, as in this case it is the substrate which carries the large electrical charge. The greatest influence of the ionic strength may be expected when the enzyme and the substrate carry the same charges at the reaction optimum, *i.e.*, when the isoelectric points of the enzyme and the substrate lie on the same side of the reaction optimum. When the enzyme and substrate carry opposite charges at the reaction optimum, it may be expected that the dependence on the ionic strength will be less, as the intermediate compound formed during the reaction by combination of the enzyme with the substrate under such circumstances will show a considerably

lower electrical charge than the two separate compounds. However, it may be that other relations then play a significant rôle, for instance, the charge of the new product formed, when the compound is split during the action of the enzyme.

Just as the ionic strength influences the different physico-chemical *properties* of the substances in solution by influencing the activity coefficients of the ions, it likewise influences the reaction velocity of the *processes* in which the ions participate (see BRONSTED (*49*)).

BRONSTED assumes that two ion species A and B must form a "collision complex" X before they can react together, X being in equilibrium with A and B, thus:

$$A+B \rightleftarrows X$$

If a small proportion of these complexes react unimolecularly to give the final products,

$$X \rightarrow C+D,$$

the velocity of the process at a given temperature must be determined by the concentration of X, when the equilibrium is reached instantaneously.

It is seen that BRONSTED'S conception of an ionic reaction corresponds quite to the reaction mechanism for an enzymatic reaction, in which also the formation of an intermediate compound between enzyme and substrate is assumed.

When the classical expression for the reaction velocity (v) is used, equation (12) is obtained, in which c signifies a concentration, and k is a constant.

$$v = k \cdot c_A \cdot c_B \tag{12}$$

If, however, the activity coefficients (f) are taken into consideration, the equation (13) is derived:

$$v = k_0 \cdot c_A \cdot c_B \cdot \frac{f_A \cdot f_B}{f_X} \tag{13}$$

hence

$$k = k_0 \cdot \frac{f_A \cdot f_B}{f_X} \tag{14}$$

where k_0 is independent of the concentration and ionic strength, which was not the case with k.

The general qualities of equation (14) are discussed by BRONSTED and terms for the positive and negative salt effect derived.

In the case of enzymes a similar treatment, yielding interesting information about enzymatic reactions, may be carried out when some simplifications are made.

Being a protein, an enzyme will always be more or less charged. This also holds true of the enzyme at its isoelectric point, where the number of the positive and negative charges are identical, thus yielding zero net charge. The relations are therefore far more complicated than in the case of simple salts.

When A is supposed to be the enzyme and B the substrate, the following may be deduced.

If the substrate, on which the enzyme acts, is an electrically uncharged molecule, the intermediate compound X will carry the same charge as the enzyme. For the uncharged substrate B the activity coefficient will be 1, while $f_A \cong f_X$, hence $k \cong k_0$. In this case therefore the ionic strength will be of no significance to the reaction velocity, and consequently no salt effect is to be expected in enzymatic processes of this kind. A very great number of enzyme reactions belong to this group, for instance, the large group of reactions produced by enzymes attacking uncharged carbohydrates.

If, on the other hand, the reaction takes place between two ions, which is the case, for instance, with the large group of proteolytic enzymes, the following three cases may be distinguished.

The first case is when the reaction takes place at the isoelectric point of either the substrate or the enzyme. In this case, say, the net charge of the substrate is zero, and this case resembles the preceding reaction concerning an uncharged substrate, *i.e.*, the reaction velocity will be more or less independent of the ionic strength. As already mentioned, an isoelectric protein may not in this respect be looked upon as uncharged. The compound X will carry the net charge of the enzyme, but it is impossible to say anything about the total number of positive and negative charges. It is easy to see that

under these circumstances it is impossible to express anything quantitatively about the relation to the ionic strength. Assuming that the same net charge of A and X will yield activity coefficients of about the same order, then $f_A \cong f_X$ and $k \cong k_0 \cdot f_B$, *i.e.*, the reaction velocity is dependent on the activity coefficient of the isoelectric substrate. As the net charge of the isoelectric substrate is zero, it may be assumed that f_B is considerably larger and nearer to one than the activity coefficients f_A and f_X for the highly charged enzyme and the intermediate compound. Under such circumstances the reaction is less dependent on the electrolyte concentration in the solution, and will only show a minor negative salt effect. Similar results are obtained if the reaction proceeds at the isoelectric point of the enzyme and not at that of the substrate. Proteolytic enzymes of the papain type probably act according to this conception.

As the second case we may take the reactions proceeding at a pH value on the same side of the isoelectric points of the substrate and the enzyme. To reactions of this kind belong the proteolytic processes due to enzymes of the trypsin type. If the isoelectric points of the enzyme and the substrate are identical, then the activity coefficients may be equal. The intermediate compound may show a net charge double that of the enzyme or substrate, and therefore its activity coefficient may be expected to be considerably smaller, *i.e.*, $f_X < f_A \cong f_B$. Hence

$$k = k_0 \cdot \frac{f_A^2}{f_X} \tag{15}$$

On increasing the salt concentration f_A (and f_B) will diminish, and f_A^2 consequently will decrease more rapidly. On the other hand, f_X is the activity coefficient of a protein molecule carrying a considerably larger number of positive and negative electrical charges than A and B, and with a net charge double that of A and B. f_X will therefore probably diminish still more rapidly than f_A (or f_B). The highest value f_X may be expected to show is the same as f_A and f_B. In this case $k = k_0 \cdot f_A$. By increasing the salt concentration k will diminish and the reaction velocity will decrease, probably rapidly, on account of the large charges of the enzyme and the substrate at the hydrogen

ion concentration in question. On the other hand, as mentioned, it must be assumed that f_X will be far smaller than f_A (or f_B) and be far more influenced by salt addition. In this case $\frac{f_A \cdot f_B}{f_X}$ will increase in value with increasing salt concentration, and consequently the possibility exists that a positive salt effect is produced and that the reaction velocity will increase with increasing salt content.

In the third case the isoelectric points of the enzyme and the substrate lie on opposite sides of the pH value of the reaction mixture. Reactions of this kind are the processes in which proteolytic enzymes of the pepsin type take part. If the pH of the solution is just the mean value of the isoelectric points, then the two proteins will carry the same net charge at this point and the intermediate complex will carry no net charge, *i.e.*, have its isoelectric point at the pH of the solution. Hence the activity coefficients of the substrate and the enzyme may be of the same order, while the activity coefficient for X may be considerably larger. If it is assumed to be approaching one, then

$$k \cong k_0 \cdot f_A \cdot f_B \cong k_0 \cdot f_A^2 \tag{16}$$

In this case there will always be a great dependence on the salt concentration, and the reaction velocity will decrease with increasing salt concentration.

From the preceding, it is evident that even this crude, pure qualitative treatment yields results of interest for the understanding of the influence of the ionic strength on enzymatic reactions, and it is highly probable that many problems with relation to the salt effect in enzymatic processes in general may be explained in this manner—by taking the interionic forces (expressed as the ionic strength) into consideration. A complete quantitative treatment of the question is not yet possible, however, due to the complexity of the reactions taking place between proteins, and to the anomalous properties of the proteins as high molecular electrolytes carrying a considerable number of both positive and negative electrical charges. Probably it may be possible to simplify the problems in some respects. For instance, the electrical charges in proteins are

removed so far from each other, that they partially react as independent charges, and thus it has been assumed by LINDERSTROM-LANG (*108*) that one z-valent protein ion makes the same contribution to the ionic strength as z univalent ions. Further the valency of protein ions may be obtained from membrane potential measurements (cf. ADAIR & ADAIR (*1*)). Also the influence of the salts on the charge of the protein molecules must be taken into consideration. However, this is not the place to extend this discussion further.

For the experimental verification of the considerations developed here, experiments may be carried out with different enzyme systems; especially those in which the isoelectric point of the enzyme (or substrate) is far removed from the pH of the reaction optimum of the process, should yield interesting results. As already mentioned the thrombin action is such a process, and it shows great dependence on the ionic strength. For theoretical reasons, however, this process is not quite satisfactory, as it includes the formation of the reaction product as an insoluble substance, and thus is not a homogeneous reaction. It seems more promising to investigate the enzymes trypsin and chymotrypsin. They show about the same reaction optimum (pH 8-9), but while the isoelectric point for trypsin is at pH 7-8, for chymotrypsin it lies at pH 5.4 (KUNITZ & NORTHROP (*106*)). Of these two enzymes chymotrypsin should show considerably greater dependence of the ionic strength than trypsin. We have therefore carried out some preliminary experiments in this direction, but as yet we have not succeeded, as the curves obtained by the method so far used give a pH optimum extending over several pH units, and thus do not allow of any satisfactory accuracy for determinations of this kind.

In this connection it is of interest to mention that the inactivation of crystalline pepsin is dependent to a very high degree on the ionic strength of the solution (STEINHARDT (*148*, *149*)).

From the considerations above it is seen that dependence on the ionic strength of the solution is a general property of enzymes—just as the dependence on the hydrogen ion concen-

tration. From these considerations, however, we may also expect that the influence of variations in the ionic strength on most enzyme reactions will be of minor importance and not comparable with the influence of variations in the hydrogen ion concentration of the solution. Probably only in certain favourable cases is the influence so great that it will be of practical importance, and the thrombin action is apparently such a process. The influence of the ionic strength on enzyme reactions must not be confused with the specific action of certain ions in some enzymatic processes as, for instance, the action of calcium ions as an activator for the formation of thrombin from prothrombin and trypsin from trypsinogen, the significance of magnesium ions for the phosphatases, or the importance of chloride for the activation of amylases. In such cases specific properties of the ions towards the enzyme in question are concerned, which are not included in the expression for the ionic strength of the salts in the solution. Naturally also the action of metal ions in enzymes containing a metal atom in the molecule falls beyond the scope of the questions discussed here; this, for instance, applies to all the respiratory enzymes containing copper or iron.

After completion of the preceding investigations an interesting paper concerning the difference in the pH at the surface of protein molecules and the pH of the solution was received (DANIELLI (*58*)). From theoretical considerations the author here predicts that the variations in the ionic strength of the solution will affect this difference, and he points out that it seems probable that the greater part of the effect of neutral salts on enzymes is due to this variation. He further emphasizes the necessity of working at constant ionic strength if the results obtained are to be compared. The experiments described on the influence of the ionic strength on the action of thrombin may be looked upon as an experimental verification of his theoretical considerations.

Probably also the phospho-esterase studied by BAMANN & SALZER (*40*) (1936) is influenced by the ionic strength. These authors find that the activity with varying pH values differs when citrate buffer containing equal concentration of citrate

ion is used instead of the usual citrate buffer of SORENSEN. By taking, as they do, equal amounts of secondary sodium citrate and adding HCl or NaOH, both the ion content and the charge of the citrate ion are changed; hence also the ionic strength is changed. This is due to the presence of polyvalent ions. The curves obtained are irregular. Probably more smooth curves could be obtained by using buffers of equal ionic strength.

In the experiments on β-glucosidases by VEIBEL & LILLELUND (*158*) (1940) the differences in pH optima in different buffers seem to be due to the specific properties of the ions in question, not to the ionic strength of the solutions. This may also be the case in the investigations on urease by HOWELL & SUMNER (*93*) (1934).

In two papers just received, BALLOU & LUCK (*38*) (1940), (*39*) (1941) describe the effect of different buffers on the activity of taka-diastase and β-amylase. As a rule they use buffers of constant ionic strength, but compare also a buffer of constant ionic strength with a buffer of constant molarity. According to our conception as presented in the preceding, such a reaction with an uncharged substrate would probably show only slight dependence on changes in the ionic strength. From the curves found by the authors just mentioned, it is also seen that a total valerate concentration (the sum of valerate ion and valeric acid) of 0.07-m and a similar buffer of ionic strength 0.05 yields equal activity at the higher pH values. At the lower values, however, they deviate, and the experiments with the mixtures with equal ionic strength show the most rapid decrease in activity. This is contrary to our results with thrombin, and may be explained by assuming a salt content to *increase* the activity of the enzyme. As has already been shown, this is not theoretically impossible for certain types of enzymes. However, in the case in question the enzyme is not of such a type and it is more likely that it is the increased content of the uncharged acid molecule in the experiments with constant ionic strength in comparison with the examples of constant total molarity which makes the difference. This conception is supported by the curves obtained with different buffers at constant ionic strength, as here the buffers, containing the

stronger, and thus more dissociated acids as phosphate and citrate buffers, show only a minor decrease in activity by increasing hydrogen ion concentration, while the weakest acids, valerate, phenylacetate, phtalate, show the largest deflection. The same explanation applies to experiments on invertase in acetate buffers described by WISANSKY (*170*) (1939), where buffers of constant ionic strength but with varying content of uncharged acid are compared.

Anomalies in buffer action in enzyme reactions on uncharged substrates were also found in the case of lipases by PLATT & DAWSON (*129*) (1925) and SOBOTKA & GLICK (*147*) (1934).

Chapter IV.

INHIBITION OF THE COAGULATION

This chapter deals with processes inhibiting the blood clotting. A considerable number of investigations of this kind have been performed by previous authors, concerning which the reader may be referred to the reviews by MORAWITZ, WÖHLISCH and OPPENHEIMER. Here regard will be paid only to papers of special significance for the problems treated.

The first section of this chapter deals with investigations on the inhibition of the blood clotting as a whole. The second section treats the inhibition of the thrombin formation, and the last section deals with the inhibition of the thrombin action.

Here only substances showing a specific inhibitory action on either the thrombin formation or the thrombin action are considered. Alteration in the medium in which the clotting proceeds are not taken into consideration; this, for instance, applies to changes in pH and in the ionic strength of the solutions used. Also substances, the action of which depends on the removal of calcium ions or prothrombin, fall beyond the scope of this chapter.

A. Inhibition of the Clotting Process as a Whole.

The inhibition of the clotting of blood plasma by means of widely different substances has been a much studied question in the history of blood coagulation, and it is easy in this way to obtain an impression of the inhibitory power of various substances. For theoretical reasons, this is a rather unsuitable method, as it is impossible to obtain sufficient information about the mechanism of the inhibition, for it may be either

the thrombin formation, the thrombin action or both processes which are acted upon by the substance in question.

For practical purposes, however, the inhibition of the clotting process as a whole is of the utmost importance, without regard to the separate reactions taking part in it. Experiments in this direction may therefore precede and supplement investigations on the two separate processes taking place during the clotting. Due to the fact that the part played by each of these reactions in the inhibitory action is not known, caution must be exercised in the deduction of conclusions of theoretical importance from such investigations. Still, with caution some suggestions may be derived, which may be useful in more thorough studies on the separate processes.

By disregarding the thrombin-inactivating properties of plasma and assuming that the inhibitory substances neutralize the thrombokinase added or a substance participating in the process corresponding in amount to the thrombokinase added —as, for instance, a thrombokinase-prothrombin complex—it is possible by comparing the clotting times obtained with the times obtained by diluting the thrombokinase to calculate the amount of thrombokinase which is able to induce clotting in the same time and therefore under these circumstances is assumed to be present in a free state in the mixture. The amount bound by the inhibitory substance added is then found by subtraction and a curve may be plotted showing the amount of thrombokinase bound in this manner by adding increasing amounts of inhibitory substance to the mixture. Such a curve was obtained in studies on the action of heparin on chicken plasma by FISCHER & ASTRUP (*78*). This curve was shown to correspond very well to the formation of a dissociable compound between heparin and the clotting substance. Increasing amounts of heparin neutralized the action of proportionally smaller amounts of thrombokinase. This result allows the conclusion that one or both of the inhibitory processes may be due to reversible reactions in the case of heparin, as heparin acts in both processes. If only one of the reactions is reversible, it will be the inhibition of the thrombin action;

the reaction being forced in this direction if this was not the case.

Experiments with chicken plasma are the most suitable in investigation of the clotting process as a whole, as no inhibitory substance has to be added in order to keep the plasma in the fluid state. So, no complications due to the presence of such substances will appear. Further, the thrombokinase employed was a very dilute chicken embryo extract, a very potent substance, which means that only very small amounts of material are added, thus reducing to a minimum the disturbing influences of additional impurities.

By investigating other substances in this manner (FISCHER & ASTRUP (*79*)) it has been shown that for the accurate determination of the strength of inhibitory substances it is necessary to use a method yielding a curve of the results, thus allowing a more accurate determination of the values, as the single determinations may vary considerably. Further, it was shown that it may be quite impossible to compare the strength of two anticoagulants—for instance, heparin and germanin—as the two curves obtained may differ in appearance, indicating not only quantitative but also qualitative differences in the action of the two substances.

By carrying the investigations further it was shown that different substances—heparin, acid and basic dyes, and salts of heavy metals—reacted differently, as some showed more dissociable compounds than others (ASTRUP (*6*)). Later it was found that curves corresponding to those obtained for substances showing no dissociation could be calculated theoretically from the equation (1) for the action of thrombokinase on plasma, thus giving some sort of confirmation of the assumptions made. For the substances assumed to yield dissociable compounds, deviations in the expected direction were found (ASTRUP & ASTRUP (*20*)).

The question is of considerable practical importance as the potency of heparin and similar anticoagulants is always measured by their inhibitory action on the clotting of whole blood or plasma. Various methods for this measurement have been

devised. The most suitable method both as regards the accuracy required and with respect to the plotting of curves, yielding not only the quantitative differences but also the qualitative, is the one based on chicken plasma described by FISCHER & SCHMITZ (*83*), which was used for the investigations just mentioned. In another paper (ASTRUP (*7*)) the standardization of heparin is treated, and it is shown that no synthetic anticoagulant is suitable as a standard for measuring heparin strength, which otherwise would be of value, as heparin, being a complicated polysaccharide derivative, can not be prepared in a sufficiently defined chemically pure state. Further, in this and another paper (ASTRUP & BEHRNTS JENSEN (*35*)) it is pointed out that the properties of the plasma system even for chicken plasma vary so much from one experiment to another that it is absolutely necessary to use a heparin standard for comparison of the preparations investigated.

According to our experiences, the chicken plasma method yields the most accurate measurements of heparin activity. Some laboratories, however, may find the method rather troublesome, and in order to find a more easy method DAM & GLAVIND (*57*) have adapted it to the use of human plasma. In this paper the authors also are aware of the qualitative differences between different anticoagulants.

It would be more convenient to use recalcified oxalated or citrated ox plasma, but so far no method is available for this. JORPES (*101*) uses whole ox blood running directly from the animal into tubes containing different amounts of the heparin solution under investigation, but this is a rather cumbersome procedure for most laboratories. We have therefore performed some experiments in order to work out a method based on ox plasma. This was done with some hope of succeeding, as it was found possible for the action of thrombokinase on ox plasma to obtain curves corresponding to the curves obtained for chicken plasma (see Chapter II).

In the following orientating experiments a purified thrombokinase from ox lung prepared as described by ASTRUP & DARLING (*24*) was used. All the substances investigated previously (ASTRUP (*6, 7*)) were studied, namely heparin, liquoid Roche,

germanin, the acid dyes chlorazol fast pink and trypanblue, the basic dyes crystal violet (methyl violet), methylene blue and Janus green and the salts $CuCl_2$ and $CdCl_2$. Further, toluidine blue and clupein sulphate were investigated because of their heparin-binding properties. Oxalated and citrated ox plasma was used, and the measurements were carried out as described previously. The optimal amount of calcium chloride solution, from 0.01 to 0.10 ml of the solution of inhibiting substance and 0.1 ml of thrombokinase are mixed in the clotting tubes. Then 1.0 ml of the plasma is added and the clotting time determined. Table XI shows some of the results obtained on oxalated plasma (with the exception of $CuCl_2$ which was obtained with citrated plasma). The clotting times are measured in seconds.

Table XI.

ml solution added	Heparin 0.05 per cent (K = 4-5)	Liquoid 0.40 per cent	Germanin 1.0 per cent	Chlorazol fast pink 0.30 per cent	Janus green 0.20 per cent	$CuCl_2$ 0.40 per cent	Toluidine blue 1.0 per cent	Clupein sulphate 1.0 per cent
0.00	24	27	21	25	21	38	21	23
0.02	27	24	22	26	25	45	29	24
0.04	27	33	22	27	29	61	31	(25)
0.06	27	46	23	32	31	82	36	(26)
0.08	30	61	24	33	29	91	37	(28)
0.10	34	(105)	23	37	31	116	43	(33)
0.15	51	—	32	52	—	—	(65)	—
0.20	>180	>240	(53)	>240	38	240	—	—

From Table XI it is seen that in most cases the interval for which reliable measurements may be carried out is too small for drawing a curve. This is especially conspicuous on comparison of the increase in clotting time from no addition to the addition of 0.10 ml inhibiting solution with the increase from 0.10 ml to 0.20 ml. For the most interesting substance, heparin, only a very small increase is found in the first interval, while in the second interval the increase is far too great to be measurable. For substances of this kind, therefore, it is not possible in this manner to devise a more simple measuring method. The limit

for the exact determination of the clotting times lies between one and two minutes. For some of the substances which, according to earlier investigations, seem to form less dissociated compounds, the results are more promising. This applies, for instance, to liquoid. Cadmium chloride precipitates the thrombokinase. Some of the dyes are used in warm solution in order to obtain an increased solubility of the dye. Clupein sulphate gives precipitate with the recalcified mixture, which makes the clotting uncertain, but it is seen that both toluidine blue and clupein sulphate even in large amounts act as inhibiting substances only to a very moderate degree.

Later it was found that some of the difficulties met with probably arise from the use of an unsuitable thrombokinase solution. The solution of ox lung thrombokinase here employed contains large amounts of partially denaturated proteins. Since it is known from the work of FISCHER (*73*) that heparin combines easily with denaturated proteins, it is no wonder that difficulties may appear. Large amounts of heparin may here be bound without interfering with the clotting and thus escape the measuring. This is of considerable importance with a substance as heparin, which acts as a potent anticoagulant even in very small amounts. When the experiments described in Chapter II with ox brain thrombokinase were finished, it was tried therefore to use such a thrombokinase which was prepared in a more cautious manner than the lung thrombokinase. The results were promising, and we succeeded in this manner in working out a method for the determination of the potency of heparin and similar substances based on ox plasma (ASTRUP & GALSMAR (*32*)). To most laboratories this method is more convenient than the chicken plasma method, and it is almost as accurate, so that it may be used for most practical purposes.

The most interesting of the inhibitory substances is heparin, which is the physiological anticoagulant produced by the organism. It was discovered by HOWELL in 1918 and later studied especially by CHARLES & SCOTT and by FISCHER & SCHMITZ. Its constitution was established by JORPES who showed it to be a mucoitin polysulphuric acid. Excellent reviews have been given on the chemistry of heparin, which therefore will not be treated here.

In two papers (ASTRUP & BEHRNTS JENSEN (*34*, *35*)) we have described the purification and investigation of heparin prepared from ox lung. Later a large amount of heparin was prepared from dog liver in order to compare it with the heparin isolated from ox lung, and it was found that it was very easy from this source to obtain very potent heparin preparations. Unfortunately, in the last steps of the purification process the whole lot was lost by accident, so that the investigation could not be completed. Recently the question has been solved by JAQUES, WATERS & CHARLES (*100*) (1942) (cf. the review of recent investigations on the chemistry of heparin by JORPES (*102*) (1943)). Crystalline barium salts of heparin from dog, ox, pig and sheep were here prepared, and the heparin from dog was found to be the most potent of the substances. The relative activities of the salts mentioned were found to be 10:5:2:1 respectively.

As a mucoitinpolysulphuric acid heparin carries a high electrical charge and it is capable of reacting with proteins and alter their properties (see especially the investigation by FISCHER (*73*)). It is possible, therefore, that heparin interferes in this manner with the blood clotting. BERGSTRÖM (*42*), one of JORPES' co-workers, therefore prepared sulphuric acid esters of various polysaccharides and investigated their inhibitory properties. None of them were found to be of a strength comparable to that of heparin, but some showed a considerable strength. He obtained the results given in Table XII for the substances used for preparing sulphuric acid esters, and the figures given for their activities correspond to the figure 11-13 for the pure heparin.

Table XII.

Substance	Activity	
	Bergström	Present Investigations
Condroitin sulphuric acid	0.2-2	—
Chitin	1.5	5
Cellulose	1	12
Pectin acid	1	—
Starch	0.5	5

In the last year we have been investigating synthetic substances prepared in a similar manner, and our results are not quite concordant with the results of BERGSTRÖM. We prepared the sulphuric acid esters of cellulose, chitin and starch and found them as measured after our methods to be considerably more potent, compared with heparin, than found by BERGSTRÖM. Our results are given in the last column of Table XII.

These results were obtained by different methods, but always with blood obtained in the usual manner from the slaughter-house. Either whole blood or recalcified plasma from oxalated blood was used. On further investigation of the problem it was found that probably the increased thrombokinase content of the blood used by us, in comparison with the blood used by BERGSTRÖM, made the difference. It was found, namely, that the synthetic polysulphuric acids under our experimental conditions acted as if they formed less dissociable compounds than heparin. Hence, with an increase of the thrombokinase content only a corresponding amount of the anticoagulant is necessary in order to prevent the coagulation, while in the case of heparin a large surplus must be taken. With small amounts of thrombokinase in the shed blood—as, for instance, under the conditions employed by BERGSTRÖM—the dissociation is of minor importance, and in this case the difference between an anticoagulant forming a dissociated compound and one yielding an undissociated compound, vanishes. When a relatively large amount of thrombokinase is present in the blood, as will be the case in blood obtained at the slaugther-house in the usual manner without special measures, the differences in the action of two such anticoagulants will increase. Whether it will be possible in this manner to explain the whole difference between our results and those of BERGSTRÖM is still an open question (ASTRUP, GALSMAR & VOLKERT (*33*)).

The same applies to our investigations on the action *in vivo* of the substances in question. With the exception of the sulphuric acid ester made from chondroitin sulphuric acid, BERGSTRÖM assumes the synthetic substances to be toxic, but he mentions only an experiment with the cellulose derivative. In a previous paper (FISCHER & ASTRUP (*80*)) it was shown that

in mouse the ingested heparin is not absorbed from the alimentary tract to any extent but is excreted unchanged with the feces in the course of about two days. In view of this finding, and as we also found the cellulose derivative toxic when given intravenously, we tried the toxicity of the substance when given by mouth. As according to the experiments with heparin, only minute amounts were supposed to be absorbed by the organism, the substance was expected to be non-toxic when given in this manner. This was also found to be the case; and this finding is of interest in view of the possible use of the substance as an anticoagulant for the preparation of blood products instead of the citrates and pyrophosphates commonly used.

The chitin derivative, however, we found to be non-toxic also on intravenous injection. So, it may therefore serve as an inexpensive substitute for heparin in the treatment of thrombotic conditions. Due to the considerable interest for such a substance we have therefore carried out extensive studies on this compound and its properties, and its pharmacology has been made the object of a careful examination by J. PIPER (*128*). All these investigations are to be published in the near future.[1])

It has already been mentioned that conclusions concerning the mechanism of the inhibitory action of anticoagulants may be drawn from experiments with whole blood or plasma only with the utmost caution, and from the experiments cited only the difference in the tendency of various anticoagulants to form more or less dissociable compounds seems to be a sufficiently established fact. In order to obtain a deeper understanding of the reactions, the two phases of the blood clotting must be investigated separately.

B. Inhibition of the Thrombin Formation.

Heparin was assumed by HOWELL to inhibit directly the transformation of prothrombin into thrombin by combining

[1]) Recent experiments by PIPER indicate that chitin polysulfuric acid is not quite as harmless against the living organism as is heparin. In a paper recently received, KARRER, KOENIG & USTERI (Helv. Chim. Acta. *26*, 1296 (1943)) describe some cellulose derivatives, which are said to be relatively non-toxic.

with prothrombin. It was therefore called an "antiprothrombin".

MELLANBY (*113*) (1935) and QUICK (*132*) (1936) found, however, that purified prothrombin solutions could be converted into thrombin without interference from heparin. At the same time FISCHER (*77*) (1936) investigated the clotting of a purified chicken fibrinogen by means of thrombokinase. In this process he found the presence of large amounts of heparin to be without any influence, but after the addition of fresh plasma or plasma heated to 56° the clotting was inhibited.

In experiments, undertaken to investigate these findings, it was now found that the fibrinogen used by FISCHER contained prothrombin, thus causing a coagulation of the fibrinogen by the addition of thrombokinase in the presence of calcium ions. Chicken fibrinogen free from prothrombin did not clot by means of thrombokinase. Further, the presence in plasma of some unknown constituent necessary for the action of heparin as an antiprothrombin was confirmed.

During our investigations a paper by BRINKHOUS, SMITH, WARNER & SEEGERS (*46*) (1939) was received, in which experiments concerning a substance (present in plasma) which acted in conjunction with heparin to prevent the conversion of prothrombin into thrombin are described. The findings of these authors were thus in accordance with ours, and a preliminary note was therefore published (ASTRUP (*14*) (1939)). This was followed by a statement by FERGUSON (*68*) (1939), who believed the results to be due to the inhibition of the thrombin formed by means of an antithrombin, thus denying the presence of an antiprothrombic substance in plasma. In an answer to this BRINKHOUS, SMITH, WARNER & SEEGERS (*47*) (1939) pointed out that the prothrombin is *not* converted into thrombin in the presence of the said substance, which therefore must be able by combining with heparin to form an *antiprothrombic* agent, even if the possibility of forming an *antithrombic* agent also exists. FERGUSON (*67*) (1939) and FERGUSON & GLAZKO (*69*) (1941) later still found that heparin does not need any co-factor for its antiprothrombic action, but as in the albumin fraction of plasma they found a substance increasing this pro-

perty, they mentioned the possibility that their solutions might contain small amounts of this substance.

Regarding the question of the action of thrombokinase on fibrinogen our results in the preparation of purified, prothrombin-free, fibrinogen solutions have already been published. Prothrombin-free chicken fibrinogen, which does not clot on addition of thrombokinase and calcium chloride was used in the investigations (*8*) and (*15*), and the purification of fibrinogen has been investigated in detail in (*26*).

As mentioned, our results concerning the antiprothrombic action of heparin confirmed the findings reported by FISCHER (*77*) and by BRINKHOUS, SMITH, WARNER & SEEGERS (*46*) but without extending them, as they merely showed qualitatively that prothrombin did not disappear in heparin plasma, as it was possible to clot such plasma by addition of thrombokinase. If thrombin eventually is formed, it is inactivated by the antithrombin present. It was therefore decided to postpone further investigations and publications, until more significant and quantitative results had been obtained. Above all, it was important to investigate the prothrombin and its properties and, if possible, to prepare purified and stable preparations. It was of equal importance to study the antithrombic effect of heparin in order to compare it with the more difficultly accessible antiprothrombic effect. While, as already mentioned, we have so far not succeeded in preparing sufficiently stable, purified prothrombin solutions, our investigations on antithrombin and the antithrombic effect of heparin have yielded results of considerable interest. The following section deals with these problems.

C. Inhibition of the Thrombin Action.

As is well known, plasma and serum contain substances which inactivate thrombin. With the discovery of heparin by HOWELL & HOLT (*96*) (1918) this question was divided into two, namely, the question about the properties and reactions of the antithrombin normally present in plasma and serum, and those of the antithrombin resulting from the addition of heparin.

HOWELL & HOLT found that heparin alone does not prevent the clotting of fibrinogen by thrombin when purified solutions are used, and this has been confirmed by several later authors. The establishment of the inhibition requires the presence of some unknown substance from plasma, and only in connection with this substance does heparin act as an "antithrombin". In order, therefore, to distinguish between the normal antithrombin of plasma and serum and the antithrombin formed from heparin, we have chosen the term "*thrombin inhibitor*" for the last-mentioned substance and retained the name "*antithrombin*" for the normal antithrombic properties of plasma and serum, as they are well known from older investigations and thus have a priority. The component in plasma then, which must be present for the formation of the thrombin inhibitor from heparin is called "*thrombin coinhibitor*".

In order to obtain reliable and, if possible, quantitative results from the experiments, it was of importance first to work out a method for measuring the normal antithrombin in plasma. The basis for this was already laid down in the preparation of purified thrombin and the establishment of a thrombin unit (T.U.).

As normal antithrombin reacts but slowly with thrombin, it is necessary to let thrombin and antithrombin act sufficiently long on each other. By most previous authors this condition has not been fulfilled, and no suitable method for the determination of the strength of antithrombin is known from the literature. It is also known that at low temperatures the interaction between thrombin and antithrombin proceeds very slowly.

We therefore decided to incubate the mixture at 37°, and at this temperature we found 15 minutes to be sufficient for neutralization of the antithrombin present in the solution. On this basis it was found possible to work out a measuring method for antithrombin of sufficient accuracy (ASTRUP & DARLING (27) (1942)). The features of the method, which made this possible, are the following:

1. Only purified antithrombin-free reagents are used for the measurements.

2. A very potent thrombin solution is used. Only in this case is it possible to say anything definite about the presence and amount of antithrombic substance, due to the complicated mechanism of the blood clotting and its sensitivity to different influences.
3. Only a fraction of the thrombin present is neutralized, so that the remaining activity may be determined with as great an accuracy as the original activity.
4. The thrombin-antithrombin mixture is incubated at 37° until full saturation.
5. The amount of thrombin solution is kept constant while the amount of antithrombin added is varied. As in reality it is the amount of active substance (thrombin) which is determined before and after neutralization, this gives the most satisfactory conditions for the measurements.
6. In order to obtain the greatest accuracy a curve is drawn from the measurements of samples containing varying amounts of the antithrombin and a corrected value is found by interpolation.

Later experiences in using this method have recently been published (ASTRUP, SELSØ & VOLKERT (*36*)). The most important observations here are that the fibrinogen employed must be prepared from tricalciumsphosphate-treated plasma (Bordet plasma) in order to give reproducible measurements, and that the thrombin preparations used for making the solutions must have a certain purity in order to give good clotting of the fibrinogen solution. Further some experiences with plasma of different species are mentioned, from which it is seen that the difficulties in the measurements vary from species to species, rabbit plasma and serum being the most satisfactory.

As a unit for antithrombin (A.T.U.) the amount of antithrombic substance was chosen, which under the experimental conditions described inactivates one unit of thrombin. The measurement is then carried out as follows:

In a series of five tubes, antithrombin-containing material is placed in amounts of 0.00, 0.05, 0.10, 0.15 and 0.20 ml, and physiological NaCl is added until 0.30 ml. Then 1.0 ml of a thrombin solution containing *a* T.U. (about 40 T.U.) per ml is

added to each tube, and they are placed in a water-bath at 37° for 15 minutes and then transferred to ice-water. In every sample the remaining thrombin content is determined in the usual manner by placing 0.10 ml of the incubated mixture in a clotting tube and measuring the clotting time after the addition of 1.0 ml of the fibrinogen solution. As a rule three determinations are made on every sample. The clotting time for the control tube containing no antithrombic substance is called t_0. The mean value of the clotting times for the tube containing n ml of antithrombic substance is called t_n. Then the amount c of antithrombin units per ml of the material in question is found from the following equation (17).

$$c = \frac{a}{n}\left(1 - \frac{t_0}{t_n}\right) \text{ A.T.U. per ml} \qquad (17)$$

The value used for t_0/t_n is found by interpolation from a curve plotted with n ml of antithrombic material as abscissa and t_0/t_n as ordinate.

We have used the method to investigate the properties of antithrombin and the amount contained in plasma and serum. In accordance with previous authors, we have found the antithrombin content of one ml of plasma or serum to be considerably larger than the amount of thrombin which may be prepared from the same quantity. Further we have found the normal antithrombin to belong to an albumin fraction in serum or plasma, as already observed by QUICK (*134*).

We have also investigated the antithrombin formed from heparin and the relation of this "thrombin inhibitor" to the normal antithrombin, for instance, by investigating the thermostability (ASTRUP & DARLING (*23, 29*)). In this manner we have found that the co-factor necessary for turning heparin into an antithrombin, which we have called "thrombin coinhibitor", is a far more delicate substance than hitherto assumed. It disappears from plasma during clotting and is therefore not found in serum, whether prepared by addition of thrombin, by spontaneous clotting or by recalcification. It is further inactivated completely by heating to 56° for 5 minutes. On the other hand, the stability of the thrombin inhibitor is more similar to that

of the normal antithrombin. While thrombin and normal antithrombin seem to unite to form an undissociable compound, called metathrombin by earlier authors, thrombin seems to react with thrombin inhibitor under the formation of a dissociable compound. The thrombin coinhibitor, like the normal antithrombin, is found in the albumin but not in the same fraction.

In a paper, just received, GLAZKO & FERGUSON (*85*) (1940) try to measure the normal antithrombin quantitatively. They call it "progressive antithrombin", while the antithrombin formed from heparin is called "immediate antithrombin". They are of the opinion that the normal "progressive" antithrombic activity is due to a proteolytic enzyme, and they therefore measure its amount by measuring the velocity of the inhibiting reaction. This of course is considerably more difficult than to determine the maximal amount of thrombin neutralized by a given amount of antithrombin, as we do, finding the antithrombin to be a definite substance which neutralizes thrombin by combining with it. With our method different physiological problems concerning the antithrombin content of the blood have been investigated in detail by VOLKERT (*159*, *160*, *161*), and his results are not always in accordance with the conceptions of previous authors working with less satisfactory measuring methods. The investigations were carried out on rabbits and have yielded interesting results. Thus the antithrombin content of the blood is found to increase during immunization with protein solutions and in obstructive jaundice. Intravenous injection of india ink prevents this increase, and lowers an increased content to a normal level. By immunization with corpuscular antigens (bacteria, blood corpuscules) no increase is obtained. On the other hand, injection of various high-molecular substances without antigenic character also gives an increase; this, for instance, is the case with gelatine, soluble starch and acacia (gum arabic). It is possible to show that also with these substances the living organism is sensitized just as in a real immunization, and that also a reaction similar to an anaphylactic shock may be produced. During this shock the antithrombin content is increased to maximum but falls in a few hours to normal level. When solutions, but not

corpuscules, are used another secondary increase is found after 24 hours, which then falls slowly in the course of several weeks. All these happenings are further shown to occur only for the antithrombic agent, which is due to heparin and the coinhibitor, while the normal antithrombic agent (the antithrombin in the stricter sense) does not change. Later VOLKERT (*162*) investigated the antithrombin content of the blood of rabbits in peptone shock. Due to the small amount of heparin produced by the rabbit, it has not been possible with earlier methods to use rabbits as experimental animals in shock, where the antithrombin or heparin content was to be studied.

Further studies deal with the antithrombin content of the blood in man under normal and pathological conditions (VOLKERT & HERTEL (*164*)). And recently it has been found both experimentally on rabbits (VOLKERT (*163*)) and clinically in man (VOLKERT & PIPER (*165*)) that a relation exists between the antithrombin content of the blood and certain thrombotic conditions, which of course is of considerable interest as a foundation for the treatment of such conditions by heparin or similar antithrombin-yielding substances.[1])

Investigations on the properties of other inhibitory substances regarding their influence on the second phase of the blood clotting, *i.e.*, in respect to their antithrombic action, are only few. This may possibly be due to the circumstance that the knowledge of the preparation of potent thrombin solutions, such as are to be used for investigations of this kind, is of a rather recent date. Investigations have been carried out by HÄUSLER & VOGEL (*91*) (1936), who find that salts of heavy metals prevent the clotting by combining with fibrinogen. Recently the question has been studied by GLAZKO & GREENBERG (*87*) (1940) and GLAZKO & FERGUSON (*86*) (1941). According to their opinion, anions act on thrombin, while cations act on fibrinogen.

We have now studied the aforementioned substances against thrombin and fibrinogen, preferably in order to see if the thrombin coinhibitor is necessary for the action of substances other

[1]) For the same purpose our method has been used by P. HOLM NIELSEN (Nordisk Medicin *29*, 2295 (1943)).

than heparin. In a clotting tube 0.10 ml thrombin is placed, and from 0 to 0.20 ml of the inhibitory solution. Then 1.0 ml of ox plasma or fibrinogen solution is added, and the clotting time is determined as usual.

The results obtained do not correspond with the previously mentioned investigations on the action of the same substances on whole plasma clotted by recalcification and thrombokinase. In several respects unexpected results are obtained. First some experiments made with heparin for comparison will be described. Solutions giving clotting times of about one minute for 0.1 ml addition are used when possible.

Heparin: A preparation with the strength K = 4—5 was used. 0.01 ml of a 0.05 per cent solution raised the clotting time of citrated oxalated plasma and Bordet plasma from about 10 seconds to a few minutes. For thrombokinase this amount was almost without any inhibitory influence, presumably due to the large amounts of denatured proteins present. The heparin solution must be diluted 10 times, namely to 0.005 per cent in order to obtain results comparable with those obtained with thrombokinase. On fibrinogen, on the other hand, 0.01 ml of a 0.05 per cent solution has only a very slight influence, for instance, from 10 seconds in the control to 12 seconds in the sample, and addition of 0.1 ml increases the clotting time only to 14 seconds. A 0.5 per cent heparin solution behaves in a similar manner. It is further found that the largest increase always happens with the first addition of heparin, and that it varies in height from fibrinogen sample to fibrinogen sample, while the later addition of large amounts of heparin is almost without any influence. This is due to small and varying amounts of the thrombin coinhibitor left in the fibrinogen solution.

Cadmium chloride and cupric chloride: A 4 per cent cadmium chloride is used, but it only prevents the clotting to a small degree in the amounts used and in plasma. Larger amounts give precipitate, and in fibrinogen solutions a precipitate is immediately formed. Therefore, it probably reacts with fibrinogen only, and to a certain degree it is prevented from doing so by the plasma proteins. The same applies to a 0.4 per cent cupric chloride solution.

Basic dyes: Methylene blue is used in a 0.8 per cent warm solution. It is without influence on plasma and inhibits only to a slight degree in fibrinogen solutions. The clotting times are difficult to determine, especially in fibrinogen solutions, due to the deep color of the solution. *Janus green* is used in a 0.2 per cent solution, and is also deeply colored. However, the fibrin threads formed adsorb the dye so that it is easy in this case to observe the clotting. It inhibits the action of thrombin in plasma. 0.10 ml raises the clotting time from about 10 seconds to about one minute. On fibrinogen it is considerably less active though not so inactive as heparin. *Methyl violet* (*crystal violet*) is

used in a 0.2 per cent solution. Both on plasma and fibrinogen it acts only to a minor extent as an inhibitory substance.

Acid dyes: Chlorazol fast pink and *trypan blue* are used, and these two substances resemble each other in their inhibitory action. On plasma 0.3 per cent chlorazol fast pink and 1.0 per cent trypan blue is used and the action is very similar to the action of heparin in a 0.005 per cent solution. But while heparin even in larger concentrations was without any significant effect on purified fibrinogen, these two dyes were more active on fibrinogen than on plasma. The solutions of the dyes must be diluted, chlorazol fast pink to 0.06 per cent, trypan blue to 0.1 per cent or lower, in order to obtain measurable clotting times not exceeding 60 seconds. Their actions are thus clearly different from the action of heparin, as they do not need the presence of a co-factor and are hampered by the presence of the plasma proteins.

Germanin and *liquoid Roche:* In plasma germanin is used in a 1.0 per cent solution, and in this concentration it is not very active. In fibrinogen solutions it must be diluted to 0.2 per cent. Similar results are obtained with liquoid, which in plasma is used in a 0.1 per cent solution, but in fibrinogen solutions may be diluted to 0.004 per cent if the addition of 0.1 ml shall give clotting times not exceeding 60 seconds. These substances therefore act similarly to the acid dyes, but liquoid seems to be a specific antithrombic substance, needing no co-factor, or it combines with fibrinogen without precipitating it.

Toluidine blue and clupein sulphate: Toluidine blue in a 1.0 per cent solution is without any significant influence on the clotting of either plasma or fibrinogen by thrombin. Clupein sulphate in small amounts in a 1.0 per cent solution lowers the clotting time of plasma and fibrinogen. Larger amounts give precipitate in plasma, but in fibrinogen solutions even very large amounts do not change the clotting time more than a few seconds.

From these results the action of inhibitory substances on plasma in the presence of thrombokinase is seen to differ from the action in the presence of thrombin; further, this again is different from the action on fibrinogen clotted by thrombin. In several cases plasma is found to hamper the action of otherwise very active inhibitory agents; this applies in particular to acid substances. In no case was a co-factor from plasma necessary to the inhibitory substances mentioned, as not one of them was increased in activity to any extent by the presence of plasma.

This, however, does not apply to the aforementioned synthetic poly-saccharide sulphuric acid esters, which act as antithrombin in a similar manner as heparin, and whose properties are to be described in a paper to be published later.

SUMMARY IN ENGLISH

After giving first a brief description of the historical development of the knowledge of blood coagulation and its present status, Chapter II deals with the activation of the coagulation by reviewing some previous works of the author and by additional experimental studies.

First the properties and the action of thrombokinase are discussed, and the relation between the clotting time and the amount of thrombokinase is examined thoroughly. Thrombokinase derived from ox brain is here studied especially, and its properties are compared with the results obtained earlier with thrombokinase from ox lung. Thrombokinase from brain is more stable than thrombokinase from lung, and a convenient method is given for the preparation of a suspension of brain thrombokinase (Q-9), which may be kept at 0° for several months without any appreciable deterioration. It is shown that qualitative differences exist between products of brain thrombokinase prepared in different ways, and that the untreated substance is the most active and stands the dilution best. Consequently, such a preparation is more suitable for investigations where a powerful thrombokinase is used—*e.g.*, for the determination of the prothrombin content of plasma. The inactivation by heating is studied, and it is found that the inactivation is not preceded by an activation, as is the case with thrombokinase from lung.

Then prothrombin and its conversion into thrombin is dealt with. First our studies on the preparation of purified and potent thrombin and its properties are reviewed, from which it is concluded that thrombin is an enzyme. Then our studies on prothrombin are reported. It was not possible to prepare as potent and stable preparations of prothrombin as of thrombin. We succeeded in showing, however, that while thrombin pro-

hably belongs to the albumins, prothrombin is a globulin. The conversion of prothrombin into thrombin therefore seems to consist in the splitting of a globulin molecule under formation of an albumin molecule. The mechanism of this process is discussed.

In the third section the so-called autocatalytic reaction during the clotting is treated. First, previously published investigations are mentioned, from which it is seen that neither the conversion of fibrinogen into fibrin nor the formation of thrombin proceeds during an autocatalytic reaction, even if the curves obtained, especially those from experiments with physical methods, seem to indicate such a reaction. Then experiments are set forth pointing at the possibility of the autocatalytic formation of an activating substance, a thrombokinase, from a precursor, a prokinase, and the probability of such a reaction is discussed.

Chapter III deals with the clotting process proper.

In the first section (A) the action of thrombin on plasma is investigated. The measurement of the potency of thrombin solutions is described. Plasma is unsuitable for such determinations and a specially prepared, ammonium sulphate-precipitated, fibrinogen is used, and comparison made with a solution of a thrombin standard. It is further shown that only on such fibrinogen solutions direct reciprocity is obtained between the amount of thrombin added and the clotting time. The curves obtained with plasma are similar to the curves obtained for thrombokinase and fit the double logarithmic equation used here.

In section B the significance of pH and ionic strength to the action of thrombin is investigated. The pH optimum for the thrombin action is studied, and it is shown that it is possible to explain the divergent results of previous authors by taking the ionic strength of the solution into consideration. In the buffers most employed for enzymatic studies the ionic strength varies with the pH of the buffer mixture, and the results obtained with such buffers differ from the results obtained with buffer mixtures of constant ionic strength. In the latter case the pH optimum is found at neutral reaction, just as by using acid or alkali for varying the pH value, while for phosphate

buffers of constant molarity the pH-optimum is found on the acid side, for ox plasma and ox fibrinogen at about pH 6.5. Neutral salts, which do not interfere otherwise with the clotting process, inhibit the action of thrombin in proportion to the ionic strength of their solutions.

In section C the species specificity of fibrinogen is investigated by obtaining curves for the pH optimum of thrombin acting on fibrinogen from various species, at either constant molarity or constant ionic strength of the buffers used. In either cases differences between the fibrinogens are found, as the pH optimum varies from species to species.

In the last section the significance of the ionic strength to enzymatic reactions in general is dealt with briefly, and the necessity of taking the salt concentration into consideration in experiments concerning enzyme reactions is pointed out. Means for climinating variations in the ionic strength are described, and some theoretical aspects of the question are discussed.

In Chapter IV the properties of the inhibitory substances and the mechanism of their action are treated.

First the clotting process as a whole is studied. This is important for practical reasons, as the activities of inhibitory substances such as heparin are measured by means of normal blood or plasma. Further the properties of such substances in whole blood or plasma determine the usefulness of the substances either for therapeutic or technical purposes. It is mentioned that different inhibitory substances act differently, not only quantitatively but also qualitatively. This must be taken into consideration on comparison of different substances, for instance, by means of curves showing the inhibitory action in relation to the concentration of the substance. According to our investigations in this respect, great differences exist between the substances investigated. This applies also to the action against thrombin, tested in plasma or fibrinogen solutions. The measurement of the heparin activity is discussed in the light of these and recent experiments.

Heparin and the synthetic polysaccharide sulphuric esters are dealt with, and it is shown that our results are not quite in accordance with the results of previous authors, neither

regarding the potency nor the toxicity of the synthetic substances. Differences in the qualitative action of these substances are described.

The action of heparin as an antiprothrombic and an antithrombic agent is discussed. In both cases a co-factor present in plasma is necessary to the inhibitory action. Our previous results concerning these questions are reviewed. Further, our method for measuring antithrombin quantitatively is presented, and different investigations on the physiology and pathology of antithrombin are dealt with.

SUMMARY IN DANISH

Efter først i Kapitel I at have givet en kortfattet Beskrivelse af den historiske Udvikling af Kendskabet til Blodets Koagulation og den nuværende Stilling, behandler Kapitel II Koagulationens Aktiveringsproces. Der refereres dels tidligere Arbejder af Forfatteren, og dels meddeles nye experimentelle Undersøgelser.

Først behandles Thrombokinasens Egenskaber og Virkning, og Relationen mellem Koagulationstiden og Mængden of Thrombokinase undersøges. Dette gælder navnlig Thrombokinase fremstillet af Oxehjerne, hvis Egenskaber sammenlignes med de tidligere for Thrombokinase fra Oxelunge fundne Resultater. Thrombokinase fra Hjerne er mere stabil end Thrombokinase fra Lunge, og en simpel Metode for Fremstillingen af en Suspension af Hjernethrombokinase (Q-9) angives. Ved 0° holder denne Suspension sig flere Maaneder uden at tabe væsentligt i Aktivitet. Det paavises, at der findes kvalitative Forskelligheder mellem Produkter af Hjernekinase fremstillet paa forskellig Maade, og at den ubehandlede Kinase er den mest aktive og kan fortyndes mest ved Maalingerne. En saadan Kinase er derfor bedst egnet for Undersøgelser, hvor en stærk Thrombokinase skal finde Anvendelse, f. Eks. ved Bestemmelse af Prothrombinindholdet i Plasma. Inaktiveringen ved Varme undersøges, og det findes, at der ikke først som ved Lungethrombokinasen gennemløbes en Aktiveringsperiode.

Derpaa behandles Prothrombinet og dets Omdannelse til Thrombin. Først beskrives vore Undersøgelser over Fremstillingen af et renset og stærkt aktivt Thrombin, og det sluttes, at Thrombinet er et Enzym. Derpaa gennemgaas vore Undersøgelser over Prothrombin. Det har ikke endnu været muligt for os at udarbejde Metoder til Fremstilling af tilstrækkeligt aktive og stabile Prothrombin Præparater. Det lykkedes dog at

vise, at medens Prothrombinet hører til Globulinerne, synes Thrombinet at høre til Albuminerne. Omdannelsen af Prothrombin til Thrombin er maaske derfor en Spaltning af et Globulinmolekyle til et Albuminmolekyle. Mekanismen for denne Proces diskuteres.

I Kapitlets tredie Del behandles den saakaldte autokatalytiske Reaktion under Koagulationen. Først omtales allerede offentliggjorte Undersøgelser, af hvilke det fremgaar, at hverken Omdannelsen af Fibrinogen til Fibrin eller Dannelsen af Thrombin sker ved en autokatalytisk Reaktion, selv om der, navnlig ved Anvendelse af fysiske Maalemetoder, opnaas Kurver, som har et autokatalyse-lignende Forløb. Derpaa beskrives Forsøg, som tyder paa, at det autokatalytiske Forløb skyldes Dannelsen af en aktiverende Substans, en Thrombokinase, fra et inaktivt Forstadium i Plasma, en Prokinase, og Muligheden for en saadan Reaktion diskuteres.

Kapitel III omhandler selve den egentlige Koagulationsproces, Fibrinogenets Omdannelse til Fibrin under Indvirkning af Thrombin.

I den første Del heraf (A) undersøges Thrombinets Virkning paa Plasma, og Maalingen af Thrombinets Styrke beskrives. Plasma viser sig uegnet til disse Maalinger, og der anvendes derfor en Fibrinogenopløsning fremstillet ved Fældning med Ammoniumsulfat. Thrombinets Styrke fastsættes ved Sammenligning med en Thrombinstandard. Kun ved Anvendelsen af en saaledes renset Fibrinogenopløsning faas der omvendt Proportionalitet mellem Thrombinmængden og Koagulationstiden. De Kurver, der faas med Plasma, ligner de tilsvarende med Thrombokinase fundne, og den dobbelt logaritmiske Ligning, der fandtes gyldig for Thrombokinasens Virkning, er ogsaa gyldig for Thrombinets Virkning.

I Afsnit B undersøges Betydningen af pH og Ionstyrken for Thrombinets Virkning. Ved at tage Hensyn til Ionstyrken er det muligt at forklare tidligere modstridende Angivelser for Thrombinets Virkning i Afhængighed af Opløsningens pH-Værdi (pH-Optimumskurven). I de Stødpudeopløsninger som almindeligvis anvendes ved Enzymundersøgelser varierer Ionstyrken med pH-Værdien, og Resultater opnaaede med saa-

danne Puffere afviger for Thrombinets Vedkommende fra Resultater fundet ved Anvendelse af Stødpudeopløsninger med konstant Ionstyrke. I sidstnævnte Tilfælde findes Reaktionsoptimet i Nærheden af neutral Reaktion, medens der med Fosfatpuffer efter SORENSEN med konstant Molaritet findes et pH-Optimum ved svagt sur Reaktion. Neutralsalte, som ikke paa anden Maade influerer paa Koagulationsmekanismen, hemmer Thrombinets Virkning i Forhold til Ionstyrken af deres Opløsninger.

I Afsnit C behandles Fibrinogenets Artsspecifitet med Hensyntagen til Ionstyrken af de undersøgte Opløsninger. Baade de Kurver over pH-Afhængigheden af Thrombinets Virkning, der findes ved konstant Ionstyrke og ved konstant Molaritet af de anvendte Stødpudeblandinger, viser, at Fibrinogener fra forskellige Dyrearter er forskellige.

I det sidste Afsnit underkastes derpaa Betydningen af Ionstyrken for enzymatiske Reaktioner i Almindelighed en kortfattet teoretisk Behandling. Nødvendigheden af at tage Ionstyrken i Betragtning ved Enzymundersøgelser paapeges, og Midler til at udelukke Ændringer i Ionstyrken angives. Nogle teoretiske Forhold vedrørende Saltvirkningen paa Enzymprocesser diskuteres kort.

I sidste Kapitel behandles de koagulationshemmende Stoffers Egenskaber og Virkning.

Først undersøges Koagulationen som Helhed. Dette er af praktiske Grunde vigtigt, da Styrken af de koagulationshemmende Stoffer, som f. Eks. Heparin, maales ved Hjælp af normalt Blod eller Plasma. Desuden er det Virkningen paa helt Blod eller Plasma, som afgør disse Stoffers praktiske Værdi enten som Lægemiddel eller til tekniske Formaal. Det findes, at de forskellige hemmende Stoffer virker forskelligt, ikke alene kvantitativt, men ogsaa kvalitativt. Dette maa tages i Betragtning ved Sammenligning af forskellige Substanser, f. Eks. ved Hjælp af Kurver over deres Virkning i Relation til Koncentrationen. Der er stor Forskel i den Henseende paa de forskellige Stoffer. Dette gælder ogsaa Virkningen overfor Thrombin (paa Plasma eller Fibrinogenopløsninger). Maalingen af Heparinets Styrke diskuteres under Hensyntagen til disse Forhold.

Heparin og syntetiske Polysakkaridsvovlsyreestere undersoges. Vore Resultater er ikke helt i Overensstemmelse med tidligere Forfatteres, hverken med Hensyn til Styrken eller Giftigheden af de syntetiske Substanser. De virker kvalitativt forskelligt.

Heparinets Virkning som et "Antiprothrombin" og et "Antithrombin" behandles. I begge Tilfælde er en Co-Faktor, som findes i Plasma, nødvendig for at Heparinet skal udvise hemmende Egenskaber. Vore tidligere Resultater vedrørende disse Spørgsmaal gennemgaas, og vor Maalemetode beskrives. De med denne udførte forskellige Undersøgelser over Antithrombinets Fysiologi og Patologi nævnes kort.

REFERENCES

1. Adair, G. S. & Adair, M. E.: Biochem. J. 1934, *38*, 199, 1230
2. Anson, M. L. & Mirsky, A. E.: J. Gen. Physiol. 1930, *13*, 121.
3. Arrhenius, S.: Immunochemie, Leipzig 1907, p. 44.
4. Arthus, M.: C. R. de la Soc. de biol. 1901, *53*, 1024.
5. — J. Physiol. Pathol. 1902, *4*, 1.
6. Astrup, T.: Enzymol. 1938, *2*, 377.
7. — Ibidem, 1938, *5*, 12.
8. — Ibidem, 1938, *5*, 119.
9. — Nature 1938, *141*, 1057.
10. — Hospitalstidende 1938, *81*, 745.
11. — Kemisk Maanedsblad 1938, *19*, 17.
12. — Ibidem, 1939, *20*, 52.
13. — Nature, 1939, *144*, 76.
14. — Science, 1939, *90*, 36.
15. — Enzymol. 1941, *9*, 337.
16. — Nordisk Medicin, 1941, *11*, 2586.
17. — Biochem. Z. 1942, *313*, 229.
18. — Dansk Tidsskrift for Farmaci, 1942, *16*, 193.
19. — Kemisk Maanedsblad, 1942, *23*, 17.
20. — & Astrup, I.: Enzymol. 1939, *6*, 64.
21. — & Darling, S.: J. Biol. Chem. 1940, *133*, 761.
22. — — Acta Physiol. Scand. 1941, *2*, 22.
23. — — Naturwissenschaften 1941, *29*, 300.
24. — — Acta Physiol. Scand. 1942, *3*, 168.
25. — — Ibidem, 1942, *3*, 311.
26. — — Ibidem, 1942, *4*, 45.
27. — — Acta Physiol. Scand. 1942, *4*, 293.
28. — — Naturwissenschaften, 1942, *30*, 63.
29. — — Acta Physiol. Scand. 1943, *5*, 13.
30. — — Ibidem, 1943, *5*, 97.
31. — & Fischer, A.: Skand. Arch. Physiol. 1936, *74*, 193.
32. — & Galsmar, I. (to be published).
33. — — & Volkert, M. (to be published).
34. — & Jensen, H. Behrnts: J. Biol. Chem. 1938, *124*, 309.
35. — — Skand. Arch. Physiol. 1938, *79*, 290.
36. — Selsø, S. & Volkert, M.: Biochem. Z. 1943, *315*, 303.
37. — & Volkert, M.: Acta Med. Scand. 1943, *115*, 393.

35. Bailey, G. A., & Lucké, J. M.: J. Biol. Chem. 1940, *135*, 111.
36. — — Ibidem, 1941, *138*, 233.
37. Bamann, E. & Salter, W.: Biochem. Z. 1936, *286*, 143.
41. Harpatt, J. O. W.: J. Physiol. 1931, *50*, 422.
42. Hedenström, S.: Z. physiol. Chem. 1936, *238*, 163.
43a. Herbrew, N. & Usnack, A.: Kgl. Danske Vidensk. Selsk. Math.-fys. Medd. 1929, *9*, No. 1.
43. Hekmeier, M.: Pflügers Arch. 1926, *213*, 642.
44. Hopansky, O.: J. Biol. Chem. 1937, *120*, 555.
45. Horder, J. & Gengou, O.: Annal. Inst. Pasteur, 1904, *18*, 98.
46. Brinkhous, K. M., Smith, H. P., Warner, E. D. & Seegers, W. H.: Am. J. Physiol. 1939, *125*, 683.
47. — — Science, 1939, *90*, 539.
48. Brodin-Salén, R.: Acta Physiol. Scand. (in press), Kemisk Manedsblad 1941, *53*, 21.
49. Brönsted, J. N.: Physical Chemistry, London 1937, p. 241.
50. Cerada, E. B.: Am. Journ. Physiol. 1926, *78*, 512.
51. Charles, A. F., Fisher, A. M. & Scott, D. A.: Trans. Roy. Soc. Can. 3. Ser. Sec. 5. 1937, *28*, 49.
52. Cohn, E. J.: J. Am. Chem. Soc. 1927, *49*, 173.
53. — Hendry, F. F. & Menkin, M. F.: J. Amer. Chem. Soc. 1928, *50*, 696.
54. Cruz, G.: C. r. Acad. Sci. 1935, *200*, 95.
55. Dam, H.: Ann. Rev. Biochemistry 1940, *9*, 362.
56. Dam, H. & Glavind, J.: Acta Med. Scand. 1938, *96*, 108.
57. — — Skand. Arch. Physiol. 1938, *82*, 221.
58. Daniélli, J. F.: Biochem. Journ. 1941, *35*, 470. (Ber. ü. die wiss. Biol. 1943, *60*, 537).
59. Denis, P. S.: Mémoire sur le sang. Paris 1859, p. 30.
60. Dyckerhoff, H.: in »Handbuch der Enzymologie«, Leipzig 1940, p. 633.
61. — & Kürten, H. F.: Biochem. Z. 1936, *284*, 111.
62. Eagle, H.: Journ. Gen. Physiol. 1935, *18*, 531, 547.
63. — J. Exper. Med. 1937, *65*, 613.
64. Fenaux, R.: Helv. Med. Acta, 1943, *10*, 3.
65. Ferguson, J. H.: Amer. J. Physiol. 1936, *117*, 587.
66. — Journ. Lab. Clin. Med. 1938, *24*, 273.
67. — Proc. Soc. Exp. Biol. Med. 1939, *42*, 33.
68. — Science 1939, *90*, 272.
69. — & Glazko, A. J.: Amer. J. Physiol. 1941, *134*, 47.
70. Fischer, A.: Biochem. Z. 1933, *264*, 169, 178, 184.
71. — Hospitalstidende, 1931, *77*, 893.
72. — Biochem. Z. 1931, *270*, 235, 250, 261, 275.
73. — Ibidem, 1935, *278*, 133.
74. — Ibidem, 1935, *278*, 320.
75. — Ibidem, 1935, *278*, 334.

76. FISCHER, A.: Biochem. Z. 1935, *279*, 108.
77. — Enzymol. 1936, *1*, 81.
78. — & ASTRUP, T.: Biochem. Z. 1935, *278*, 326.
79. — — Proc. Soc. Exp. Biol. Med. 1938, *38*, 494.
80. — — Proc. Soc. Exp. Biol. Med. 1939, *42*, 81.
81. — & HECHT, E.: Biochem. Z. 1934, *269*, 115.
82. — & HERRMANN, H.: C. r. Lab. Carlsberg, 1937, *22*, 169.
83. — & SCHMITZ, A.: Z. physiol. Chem. 1932, *210*, 129.
84. FULD, E.: Hofmeisters Beiträge chem. Physiol. 1902, *2*, 514.
85. GLAZKO, A. J. & FERGUSON, J. H.: J. Gen. Physiol. 1940, *24*, 169.
86. — — Amer. J. Physiol. 1941, *134*, 54.
87. — & GREENBERG, D. M.: Ibidem, 1940, *128*, 399.
88. GREEN, A. A.: J. Amer. Chem. Soc. 1933, *55*, 2331.
89. HAMMARSTEN, O.: Z. physiol. Chem. 1899, *28*, 98.
90. — Lehrbuch d. physiol. Chem. 1926, p. 204.
91. HÄUSLER, H. & VOGEL, L.: Biochem. Z. 1936, *287*, 405.
92. HERBERT, F. K.: Biochem. J. 1940, *34*, 1554.
93. HOWELL, S. F. & SUMNER, J. B.: J. Biol. Chem. 1934, *104*, 619.
94. HOWELL, W. H.: Arch. Internal. Med. 1914, *13*, 76.
95. — Physiol. Rev. 1935, *15*, 435.
96. — & HOLT, E.: Amer. J. Physiol. 1918, *47*, 328.
97. HUDEMANN, S.: Kolloid Zeitschr. 1940, *92*, 189.
98. HUISKAMP, W.: Z. physiol. Chem. 1905, *44*, 182.
99. JAQUES, L. B.: J. Physiol. 1942, *100*, 275.
100. — WATERS, E. T. & CHARLES, A. F.: J. Biol. Chem. 1942, *144*, 229. (Chem. Zentralbl. 1943 I, 1784).
101. JORPES, E.: Biochem. Journ. 1935, *29*, 1817.
102. — Z. physiol. Chem. 1943, *278*, 7.
103. KIRBY, A. H. M. & NEUBERGER, A.: Biochem. J. 1938, *32*, 1146.
104. KUGELMASS, I. N.: C. r. Soc. Biol. 1922, *87*, 802.
105. — Arch. intern. Physiol. 1923, *21*, 139.
106. KUNITZ, M. & NORTHROP, J. H.: J. Gen. Physiol. 1935, *18*, 433.
107. KUWASHIMA, K.: Journ. Biochem. (Jap.) 1923, *3*, 91.
108. LINDERSTRØM-LANG, K.: Trans. Faraday Soc. 1935, *31*, 324.
109. LUNDSTEEN, E.: Undersøgelser over Mælkens Koagulation. Diss. Copenhagen 1936.
110. MELLANBY, J.: Journ. Physiol. 1909, *38*, 28.
111. — Proc. Roy. Soc. Lond. Ser. B, 1930, *107*, 271.
112. — Ibidem, 1933, *113*, 93.
113. — Ibidem, 1935, *116*, 1.
114. MERTZ, E. T. & OWEN, C. A.: Proc. Soc. Exp. Biol. Med. 1940, *43*, 204.
115. — SEEGERS, W. H. & SMITH, H. P.: Ibidem, 1939, *42*, 604.
116. MICHAELIS, L.: J. Biol. Chem. 1930, *87*, 33.
117. — Biochem. Z. 1931, *234*, 139.
118. — & MENDELSSOHN, A.: Biochem. Z. 1913, *58*, 315.

119. Michaelis, L. & Rona, P.: Biochem. Z. 1910, *27*, 38.
120. Mills, C. A.: J. Biol. Chem. 1921, *46*, 135, 167.
121. — & Gurst, G. M.: Amer. J. Physiol. 1921, *57*, 395.
122. Morawitz, P.: Ergebn. Physiol. 1905, *4*, 307.
123. Northrop, J. H.: Journ. Gen. Physiol. 1938, *21*, 335.
124. Oppenheimer, C.: »Die Fermente«, 1926, Hauptwerk.
125. — »Die Fermente«. Supplem. II, Haag, 1939, p. 966.
126. — Ibidem, p. 987.
127. Panum, P. L.: Om Fibrin i Almindelighed og om dets Koagulation i Særdeleshed. Diss. Copenhagen, 1851.
128. Piper, J.: (to be published).
129. Platt, B. S. & Dawson, E. R.: Biochem. Journ. 1925, *19*, 860.
130. Plum, P. & Larsen, Hj.: Z. klin. Med. 1941, *139*, 666.
131. Quick, A. J.: Amer. J. Physiol. 1935, *114*, 282.
132. — Proc. Soc. Exp. Biol. Med. 1936, *35*, 391.
133. — Amer. J. Physiol. 1936, *115*, 317.
134. — Amer. J. Physiol. 1938, *123*, 712.
135. — Science 1940, *92*, 113. (Chem. Zentralbl. 1941 I, 938).
136. — Stanley-Brown, M. & Bancroft, F. W.: Amer. Journ. Med. Sci. 1935, *190*, 501.
137. Seegers, W. H.: J. Biol. Chem. 1940, *136*, 103.
138. — Smith, H. P., Warner, E. D. & Brinkhous, K. M.: J. Biol. Chem. 1938, *123*, 751.
139. Smith, H. P., Warner, E. D. & Brinkhous, K. M.: Amer. J. Physiol. 1934, *107*, 63.
140. Schmitz, A.: Z. physiol. Chem. 1933, *222*, 155.
141. — & Fischer, A.: Ibidem, 1933, *216*, 264.
142. Schütz, E.: Ibidem, 1885, *9*, 577.
143. — & Huppert: Pflügers Arch. 1900, *80*, 470.
144. Selse, S.: Nordisk Medicin, 1943, *19*, 1314.
145. Silberberg, M.: Physiol. Rev. 1938, *18*, 197.
146. Sjöqvist, J.: Skand. Arch. Physiol. 1895, *5*, 317.
147. Sobotka, H. & Glick, D.: Journ. Biol. Chem. 1934, *105*, 221.
148. Steinhardt, J.: Nature 1936, *138*, 74.
149. — Kgl. Danske Vidensk. Selsk. Skrifter. Math.-fys. Medd. 1937, *14*, Nr. 11.
150. Strombeld, H.: Biochem. Z. 1911, *37*, 177.
151. Svedberg, The & Pedersen, K. O.: The ultracentrifuge. Oxford 1940.
152. Thorbjarson, O.: Nature 1940, *145*, 305.
153. — Acta Med. Scand. 1940, *104*, 291.
154. — Prothrombin hos sunde og syge. Diss. Aarhus 1941.
155. Tiselius, A.: Biochem. Journ. 1937, *31*, 1464.
156. Tsunoo, S.: Pflügers Arch. 1924, *205*, 255.
157. — Ibidem, 1925, *210*, 331.
158. Vebell, S. & Lillelund, H.: Enzymol. 1940, *9*, 161.

159. VOLKERT, M.: Biochem. Z. 1941, *309*, 337.
160. — Acta Physiol. Scand. 1942, *5*, Suppl. No. 15. Diss. Copenhagen.
161. — Biochem. Z. 1943, *314*, 34.
162. — Acta Physiol. Scand. 1943, *5*, 365.
163. — Acta Chir. Scand. 1943, *89*, 379.
164. — & HERTEL, E.: Ugeskrift for Læger 1943, *105*, 782.
165. — & PIPER, J.: Acta Chir. Scand. 1943, *89*, 417.
166. WARNER, E. D., BRINKHOUS, K. M. & SMITH, H. P.: Amer. J. Physiol. 1936, *114*, 667.
167. — — — Ibidem, 1939, *125*, 296.
168. WEITNAUER, H., GRÜNING, W. & WÖHLISCH, E.: Biochem. Z. 1941, *307*, 325.
169. WIDENBAUER, F. & REICHEL, CH.: Ibidem, 1941, *309*, 100, 415.
170. WISANSKY, W. A.: J. Amer. Chem. Soc. 1939, *61*, 3330.
171. WÖHLISCH, E.: Ergebn. Physiol. 1929, *28*, 571.
172. — Ibidem, 1929, *28*, 443.
173. — Ibidem, 1940, *43*, 174.
174. — Naturwiss. 1936, *24*, 513.